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BIOLOGICAL BULLETIN

VARIATION OF NORMAL GERM CELLS. STUDIES IN AGGLUTINATION.

A. J. GOLDFORB,

FROM THE COLLEGE OF THE CITY OF NEW YORK.

It has been generally assumed that eggs or sperm, freshly shed from different echinid individuals at the height of the breeding season, are in essentially the same physiologic condition. This assumption has led to gross errors, discordant experimental results and unnecessary confusion.

Nearly everyone who has made prolonged experimental studies with echinoderm eggs or sperm has recorded evidences of a disturbing variability, even in freshly shed germ cells. There has been, however, little or no agreement concerning (1) the exact physiologic condition at the time of maturing or shedding, (2) the extent of the change or changes at fertilization; whether these changes are in one direction or cyclical, (3) the effect of such changes upon the behavior of the fertilized eggs, (4) the number and kind of changes, and (5) the underlying causes of these changes.

In respect to sperm it has also been assumed that they are relatively constant when shed.

As early as 1883 Born noted that the eggs in the last part of the egg string (*Bufo cinereus*) developed irregularly or did not develop at all. Koehler (1915) made a similar observation for echinid eggs, *i.e.*, eggs were not in the same physiologic state when shed, for those nearest the exit pores were the oldest. Lillie, R. S. (1908, 1915) observed that the age of the eggs (starfish) was an important factor in their subsequent behavior. He corroborated Gemmill (1900) that the eggs changed with age, improving at first and subsequently deteriorating. Morgan [1]

[1] Reviewed in his "Experimental Embryology" (1927).

noted that the eggs (*S. purpuratus*) showed signs of ageing even when in the ovary, for the jelly layer was reduced as in eggs that were aged for several days in the laboratory.

Marked changes have been noted towards the close of the breeding season. Gemmill (1900) among others noted that at this time the echinid eggs lived a shorter time, polyspermy occurred more and more frequently, fertilization was delayed, and sperm lived a shorter time. Heilbrunn (1915) recorded that the concentration of sperm (*Arbacia*) required to elevate the fertilization membrane was greater towards the close of the breeding season, and that there was progressively greater difficulty of the eggs to elevate the membrane. Hyman (1923) also noted changes in the viscosity of ageing eggs.

Lillie, F. R. (1914) concluded that "the condition of the gonads is the most variable thing in summer sea urchins . . ." and in 1915 concluded that the large variations (*Arbacia*) were due to the use of germ cells after the height of the breeding season that variations prior to this time are due to faulty technique, for with proper precautions "both eggs and sperm are relatively constant."

Goldforb (1917, 1918) studied the variability of eggs of three species of echinoderms, *Toxopneustes*, *Hippanoë*, *Arbacia*, studied the variation in size, color, shape, amount of jelly, rate and manner of membrane formation, rate and numbers of fertilized eggs, rate and regularity of cleavage. These studies were made during three seasons. He concluded that freshly shed eggs at the height of the breeding season were not constant, that they showed wide ranges of variability, and sometimes even extreme states of deterioration. He concluded that varying degrees of overripening occurred prior to shedding.

Just (1919) came to a similar conclusion after studying *Echinarachnius* eggs. He records a wide variation due largely to corresponding degrees of deterioration. He also concluded that "ovary" eggs (of *Asterias* also) were more variable and inferior to shed eggs, due to underripeness. Graves (1928) found that *Cummingia* eggs behaved as echinid eggs described by Goldforb, namely, that deterioration occurred before spawning, with a corresponding wide range in the vitality of the eggs. Gee (1916)

for *Fundulus* eggs, Calkins (1920) for *Uroleptus*, attributed variations to corresponding internal differences.

Other evidence of changes in the egg, prior to shedding, is afforded by the work of Hertwig (1906, 1907), Kuschekewitch (1910), and Riddle (1914), that overripeness of the eggs decreased developmental energy, and increased the proportion of surviving males. Koehler (1915) noted the tendency of overripe germ cells (echinid) to produce more matroclinous larvæ. Hertwig (1890), Grief (1901), Newman (1921), and others attributed the increase in natural parthenogenesis to ageing of eggs. Fuchs (1914) and East (1915, 1917, 1918) recorded the increase in self fertilization with overripeness of the germ cells.

These citations may serve to indicate the background for the present study of the variation of germ cells, and of the subsequent studies of ageing germ cells.

The present study gives evidence of a surprisingly wide range of variation, not only among eggs, but also among sperm, when these germ cells were freshly collected, freshly shed, and freshly tested, at the height of the breeding season. *These observations emphasize the need, in experimental work, of determining by simple tests, the exact physiologic condition of the eggs and of the sperm of each individual.* By selecting germ cells in approximately the same physiologic condition, one may minimize the disconcerting differences in experimental results.

The experiments were performed in 1924 and in 1926 at the Marine Biological Laboratory at Woods Hole. My thanks are due the Directors for the facilities of the Laboratory.

Technique.

Because so much depends upon the exact details of technique, and to avoid repetition in subsequent studies, a concise statement is here given of the experimental procedure. This is primarily the procedure of Lillie, F. R. (1914). *Arbacia punctulata were brought directly from the collecting boat to the laboratory and immediately tested.* Eggs that spontaneously flowed from the aboral openings are called "shed" eggs. When ovaries were removed as intactly and gently as possible, the eggs thus liber-

ated are termed "ovary eggs." [1] Similarly "shed" and "testes" dry sperm were obtained. The germ cells from each individual were kept separately. Eggs were washed in 300 cc. or more sterile sea-water, and the final egg to water volume was usually 1 to 3. The eggs were gently shaken every ten minutes for one hour. The supernatant or "egg water" was removed, *examined for unripe, ripe, and overripe eggs, and for jelly. If present, they were immediately removed.* Their presence would alter the subsequent agglutination reaction. The "egg water," the solutions therefrom, and the dry sperm, were kept under wet cloths to minimize evaporation and to maintain a lower temperature, 20° C.[2] Capillary mouth pipettes were sterilized (against sperm and agglutinins) in a jar of 8,000 cc. tap water, then rinsed in 3,000 cc. sterile sea-water, then used in transferring the "egg water" solution to the sperm suspension on the slide. Each test was made with a different pipette.

After all the "egg water" solutions were made, each with a different pipette, a 1 per cent. sperm suspension was prepared, two measured drops placed on the slide, and the "egg water" solution gently blown under the raised cover slip. The reversible agglutination of the sperm was measured in seconds. A "loud timer" aided in this.

Sea water was carefully collected in glass at the incoming high tide, filtered, and stored in glass for 3 to 10 days before using, and evaporation minimized, by covering with wet cloths. This sterile sea water was used in all experiments at approximately 20° C.

Experimental Error.

The accuracy of the technique was determined in two ways:

1. *Repetition Test.*—The agglutination test was immediately repeated with other samples of the same solution.

2. *Aliquot Part Test.*—Eggs were divided into two equal parts in similar volumes of sea water and similar egg to water dilution. The egg water of each was then tested, one immediately after the other.

[1] The term "ovary eggs" is used by Just (1919) and myself differently, with correspondingly different results. Just cut the ovaries into pieces and presumably liberated many unripe eggs. In my experiments the ovaries are barely injured, with liberation of minimal number of unripe eggs.

[2] Temp. 20°–22° C. in different experiments.

These two tests were made in nearly every experiment. Any variation in these tests was considered a measure of the experimental error.

Out of 19 such tests the agglutination time was exactly the same in 8 tests. In 6 tests there was a difference of but 1 second. In 1 test the difference was 3 seconds and in 1 test the difference was 4 seconds. *The average difference was 1 second, or 4.5 per cent.* This may be considered the experimental error.

Variation in eggs and in sperm from different individuals was then studied.

The Variation in Freshly Shed Eggs from Different Individuals.

Table I gives the observations in 19 experiments, including 58 females tested separately, 2 to 4 in each experiment. These experiments were made from July 1 to 26. In some experiments the egg to water ratios are not the same for all the females. Hence these ratios are given in each instance so that corrections may be made. A fresh sperm suspension was made for testing the egg waters of two to four females of each series. The table gives the observed agglutination time for each egg water, the calculated agglutination time,[1] the difference or variation in seconds and in per cent.

The freshly collected, freshly shed, and freshly tested eggs in each series varied from 2 to 55 seconds calculated time. The variation was least in Experiments 12, 20 and 26, where the different females differed by 2, 2, and 4 seconds respectively. This is within the experimental error.[2] In 10 experiments the variation was 5 to 10 seconds; in 4 experiments the variation was 11 to 20 seconds; in 1 experiment the variation was 30 seconds, and in 1 experiment the variation was 55 seconds. The average variation was 12.0 seconds. The variation in control experiments ranged from 0 to 4 seconds only, with an average of only 1 second.

The minimal variation was 9 per cent. in 1 experiment. In 3 experiments the variation ranged from 16 to 26 per cent., in 4

[1] Calculated for differences in egg to water ratio.

[2] The percentage differences, 9, 19 and 26 per cent., are however much greater than the maximal experimental error.

experiments from 37 to 45 per cent., in 6 experiments from 66 to 87 per cent., in 3 experiments from 100 to 120 per cent., in 1 experiment 400 per cent., and in 1 experiment 1,300 percent. These averaged 142 per cent. The expermental error averaged 4.5 per cent. *The "best" eggs in each day's collecting at the height of the breeding season varied by as much as 1,300 per cent.*

TABLE I.

SHOWING WIDE VARIATION IN AGGLUTINATION TIME WHEN "NORMAL" EGGS FROM FRESHLY COLLECTED, FRESHLY EXAMINED FEMALES ARE TESTED BY THE SAME SPERM SUSPENSION.

Exp. No.[1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
1	1	55	1 : 3	1/100				
	2	27	1 : 3					
	3	25	1 : 3					
	4	25	1 : 3		30	120		
2	1	12	1 : 4	1/60				
	2	35	1 : 8				55	400
3	1	9	1 : 5	1/60				
	2	12	1 : 6					
	3	23	1 : 2				9	100
4	1	9	1 : 5	1/120				
	2	27	1 : 2				10	110
5	1	13	1 : 8	1/160				
	2	15	1 : 8					
	3	8	1 : 8					
	4	23	1 : 4				7	87
6	1	17	1 : 3	1/80				
	2	13	1 : 3					
	3	22	1 : 3		9	69		
8	1	24	1 : 3	1/160				
	2	14	1 : 3					
	3	23	1 : 3		10	71		
10A	1	0	1 : 6	1/320				
	2	13	1 : 6					
	3	10	1 : 6		13	1,300		

Average experimental error—1 second or 4.5 per cent.

Average variation in "normal" fresh eggs—12.0 seconds or 142 per cent.

[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

TABLE I.–(Continued)

Exp. No.[1]	♀ No.	Agglutination Time in Sec.	Egg to Sea-water Ratio.	Egg Water Dilution.	Difference in		[2] Calculated Difference in	
					Seconds.	Per cent.	Seconds.	Per cent.
10B	1	23	1 : 3	1/80				
	2	26	1 : 3					
	3	20	1 : 5					
	4	19	1 : 3				7	37
11	1	13	1 : 3	1/320				
	2	16	1 : 3					
	3	14	1 : 5					
	4	15	1 : 3				6	45
12	1	12	1 : 6	1/320				
	2	13	1 : 6					
	3	11	1 : 6					
	4	15	1 : 4				2	19
14	1	29	1 : 2	1/320				
	2	21	1 : 2		8	39		
19	1	50	1 : 3	1/320				
	2	30	1 : 3		20	66		
20	1	23	1 : 5	1/320				
	2	30	1 : 3				2	9
22	1	21	1 : 3	1/300				
	2	26	1 : 3					
	3	18	1 : 3		8	44		
23	1	19	1 : 3	1/300				
	2	18	1 : 2					
	3	19	1 : 3				6	16
24	1	17	1 : 3	1/300				
	2	29	1 : 3					
	3	28	1 : 3		12	70		
25	1	15	1 : 3	1/300				
	2	16	1 : 3					
	3	22	1 : 4					
	4	16	1 : 3				11	77
26	1	13	1 : 2	1/300				
	2	15	1 : 3					
	3	10	1 : 5				4	26

Examination in detail of a few examples may make more clear this extraordinary range in variability. In Experiment I the agglutination was 25 seconds for each of 2 females, 27 seconds and 55 seconds for the other two females with the same egg to water volumes, the same dilution of egg waters, the same sperm

[1] These numbers are also the dates in July when each experiment was performed.

[2] To correct for differences in egg to water ratio.

suspension. Lillie (1914) showed that a more exact procedure consists in finding a dilution of egg water in which the agglutination time is about 8 seconds. When higher concentrations of egg water are used, the agglutination values are not so exact. This is admitted. The necessity for rapid tests with the different suspensions and solutions necessitated the use of a low concentration of egg water but not necessarily the lowest that would give an 8 second agglutination. The fact that the same concentrations were used throughout makes the results comparable, and the error too small to materially affect the results.

In Experiment 8 the agglutination time for the eggs of the 3 females was 24, 14, and 23 seconds respectively. In Experiment 10*A* one female registered 13 seconds, another 10 seconds, *and a third did not agglutinate at all.* In Experiment 22, the values were 18, 21, and 26 seconds respectively.

It should be recalled that this variation occurred in freshly collected urchins, the "best" of the day's collection, at the height of the breeding season, and that the eggs and sperm were freshly shed, immediately after arrival from the collecting boat. These are "normal" eggs. These should register the minimal variation. They actually register a variation from 2 to 1,300 per cent.

In Experiment 19, with a 1/320 egg water dilution, the agglutination time of the eggs of female 1 was 50 seconds. Under exactly the same circumstances the eggs of female 2 registered only 30 seconds. I do not interpret this to mean *that the eggs of female 2 were in the same degree of ripeness as those of female 1, secreting only 3/5 as much agglutinin as female 1.* My interpretation is that in addition to an uncalculable but relatively small genetic difference in agglutinin production [1] *the large difference is due to the greater overripening of the eggs of female 2 prior to shedding.* The evidence in support of this interpretation will be given later. In Experiment 10*A*, with egg water dilutions the same, female 2 registered 13 second agglutination. These fresh "normal" eggs were by a variety of tests shown to be in relatively poor condition, *i.e.*, overripe when shed. The eggs of female 3

[1] Loeb and Chamberlain (1915) measured differences in enzyme content of eggs (*Arbacia*) but whether these differences were genetic, as assumed, or due to ageing, is not clear.

were in still poorer condition, *i.e.*, more overripe, registering only 10 second agglutination. The eggs of equally fresh "normal" eggs of female 1 were extremely deteriorated, giving rise to no agglutination at all.

A much larger number of experiments were made than those listed in Table I. The 19 listed are one series representative of the unexpected, consistent and large variation in freshly shed or "normal" eggs of *Arbacia*.

It might be objected that the calculated differences, to correct for differences in egg to water ratios, are only approximate. But in the experiments where no such calculation was necessary, because all egg to water ratios were the same, the variation was practically as large, namely, 6, 6, 19, 23, 30, 37, 39, 44, 66, 69, 70, 71, 110, 130 per cent.

This extraordinarily large variation in agglutination time closely corresponds with equally large variations in size, color, shape of eggs, thickness of jelly layer, fertilizability, rate of membrane formation, rate, regularity, and per cent. of cleavage. These latter variations have been shown (Goldforb, '18*a*, 18*b*) to represent corresponding degrees of ageing or deterioration. And it is presumptive and later will be demonstrated in detail that the differences in agglutination time also measure degrees of deterioration.

Variation in Freshly Shed Sperm from Different Individuals.

It is concluded that at the height of the breeding season (1) the egg is the actively changing cell, secreting varying amounts of agglutinin; (2) that agglutination time is proportional to the quantity of agglutinin thus liberated. The question arose whether the sperm is a biologic constant, or varies as the egg does.

The following experiments (with those in subsequent studies) demonstrate that *freshly shed sperm from different males are just as widely variable as freshly shed eggs.*

Some typical experiments are brought together in Table II. In Experiment 9, for example, samples of the egg water of female 1 were tested separately by freshly prepared sperm suspensions of

three males. The agglutination was 10, 0, and 9 seconds respectively. When fresh suspensions of the same three males were tested with the egg water of female 2, the results were quite different, namely 17, 0, and 13 seconds, respectively. The 0 denotes no agglutination. Fresh suspensions from the same three males against egg water from female 3 gave even larger agglutination values, namely, 23, 0, and 13 seconds respectively.

TABLE II.

SHOWING WIDE VARIATION IN AGGLUTINATION TIME WHEN "NORMAL" *i.e.* FRESHLY SHED SPERM FROM DIFFERENT MALES ARE TESTED BY THE SAME EGG WATER.

Exp. No.[1]	♀ No.	♂ 1	♂ 2	♂ 3	♂ 4	Difference in	
						Seconds.	Per cent.
9	1	10	0	9		10	1,000
	2	17	0	13		17	1,700
	3	23	0	13		23	2,300
Aver.		17	0	11			
10	1	15	33			18	120
	2	7	22			15	214
	3	12	17			5	41
13	1	15	14	12		3	25
	2	13	8	12		5	62
	3	13	13	10		3	30
	4	13	11	18		8	63
11	1	12	0	12	9	12	1,200
	2	11	0	16	15	16	1,600
	3	25	0	33	9	33	3,300
	4	10	6	18	15	12	200
	5	17	0	22	13	22	2,200
Aver.		15.0	1.2	20.2	12.2		

[1] These numbers are also the dates in July when each experiment was performed.

It should be noted that the *sperm of male 1 gave consistently the longest agglutinations, with all three females. Male 3 gave intermediate values. Male 2, though freshly shed and "normal", did not agglutinate in any egg water.* The average agglutination time for male 1 was 17 seconds, for male 3, 11 seconds, for male 2, none. Male 1 gave 54 per cent. longer agglutination reactions than male 3, and 1,700 per cent. longer than male 2. If male 2,

whose sperm were not agglutinated in any of the tests, be omitted, the variation is 1, 4, and 10 seconds, or 11, 30, and 77 per cent.

It is also possible by these tests to pick out which eggs are most potent. Female 3 gave the longest agglutination reactions. Female 1 gave the briefest and female 2 intermediate values.

A given sperm suspension gave different values with eggs from different females. Likewise eggs from one female gave different values with sperm from other males. But eggs or sperm of a given individual gave the same relative values with other germ cells.

In Experiment No. 9 an extraordinarily wide difference in agglutinability occurred in the sperms of the three males. *The variation is as great as among eggs from different females. Agglutination time appears to be dependent upon the physiologic condition of the eggs as well as upon the condition of the sperm at the time of testing.* The next study will consider this in detail.

Other experiments gave essentially similar results. In Experiment 10, 2 males were tested separately against 3 females. Male 2 gave consistently longer agglutinations than male 3, namely, 41, 120, and 214 per cent. respectively. In Experiment 13 the sperm of 3 males were tested separately against the eggs from 4 females. The agglutination time varied by 25, 30, 62, and 63 per cent. respectively. In Experiment No. 11, 4 males were tested against 5 females. Male 3 gave consistently longest agglutination values, with an average of 20.2 seconds. Male 1 averaged 15.0 seconds, male 4 averaged 12.2 seconds, and male 2 gave an average of only 1.2 seconds. This male gave no agglutination in 4 out of 5 females. If male 2 be ignored, the difference in agglutinability of these males was 33, 45, 69, 80, and 266 per cent. If male 2 be included, the differences were 1,200, 1,600, 3,300, 200, and 2,200 per cent. The dry sperm of these 4 males had been recorded at the time of shedding as follows: No. 3 best, No. 1 good, No. 4 poor. Male 2 was not recorded. This is in very close agreement with their agglutinability.

The results are unmistakable. The sperm that gives high agglutination values with 1 female tends to give high, though not the same, values with other females. Vice versa, sperm

that gives low agglutination values with one female gives consistently low but not necessarily the same values with other females. The differences with a given sperm are due primarily to differences in the physiologic condition of the eggs of the different females. The reverse is also true, *i.e.*, when a given egg water is tested by different males, the differences observed denote primarily differences in the physiologic condition of the different sperms.

It is evident that the *agglutination time of freshly shed "normal" germ cells is dependent not only upon the condition of the eggs but also upon the condition or agglutinability of the sperm.* Not only do freshly shed eggs from different individuals vary very widely in their ability to agglutinate a given sperm, but a given freshly shed sperm varies as widely in agglutinability, with eggs from different females.

The cause or causes of this variability in sperm will be discussed in the next study.

Variability of Shed Versus Ovary Eggs.

It seemed worth while to compare the agglutination time of "shed" versus "ovary" eggs. It should be recalled that these "ovary eggs" come from females whose *ovaries were removed as intactly and as gently as possible.* These ovary eggs include the minimum of unripe eggs. Such ovary eggs will differ therefore from those described by Just (1919) in which ovaries were cut into pieces, thus liberating many unripe eggs.

For purposes of comparison all experiments, in which the same egg water concentration was used, are brought together in Table III. The eggs of all females in an experiment were tested by the same sperm in fresh suspensions.

Table III indicates that "ovary" eggs tended to give longer agglutination values and a wider range of variability than shed eggs. In the 8 experiments involving 22 females, the "ovary" eggs in each series varied by 1, 4, 5, 7, 8, 8, 12, and 20 seconds respectively. The average was 8.1 seconds. In the 6 experiments including 20 females, the "shed" eggs in each series varied by 1, 1, 3, 4, 6, and 13 seconds. The average was 4.6 seconds. (If female 1 in Experiment No. 9 be omitted, because

no agglutination occurred, the variation among the "shed" eggs would be 1, 1, 3, 3, 4, and 6 seconds, or 3 seconds average.) Experiment 2 is especially interesting because the shed and the ovary eggs were tested by samples of the same sperm. The shed eggs gave 11 and 12 seconds respectively, while the ovary eggs in the same egg water concentration gave much longer values, *viz.*, 15 and 35 seconds respectively.

TABLE III.

COMPARISON OF "SHED" AND "OVARY" EGGS IN 1/320 EGG WATER DILUTION. EACH EXPERIMENT TESTED BY THE SAME SPERM SUSPENSION. FIGURES DENOTE AGGLUTINATION TIME IN SECONDS.

Exp. No.[1]	Shed Eggs.				Variation in Sec.	Aver. Aggl. Time.	Ovary Eggs.				Variation in Sec.	Aver. Aggl. Time.
	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4		
2	11	12			1	11.5	15	35			20	25
5							8	9	6	13	5	9
9	0	13	10		13	7.7						
10	12	13	11	15	4	12.7						
11	13	16	14	15	3	14.5						
12	14	9	15	12	6	12.5						
14							29	21			8	25
19							28	27			1	27.5
20							23	30			7	26.5
22							22	26	18		8	22
23	20	19	20		1	14.7						
24							17	29	29		12	25
25							16	16	22	16	4	17.5
Average					4.6	13.2					8.1	20.7

[1] Numbers correspond with dates in July when experiment was performed.

The larger variability among "ovary" than among "shed" eggs is attributed to the presence of a larger proportion of overripe eggs. This will be discussed in the next study. More intensive study needs to be made of "ovary" eggs, with a definite knowledge of the relative numbers of unripe, ripe, and overripe eggs, and the degree of overripeness.

SEASONAL VARIATION.

In the first half of July, 1926, nearly all the *Arbacia* gave numerous shed eggs. In the second half of the month there were very much fewer shed eggs and more "ovary" eggs. The

same observations were made in July, 1924. The eggs in the first half of the month were mostly "good" ones, *i.e.*, in good physiologic condition, while those in the second half tended to be "poorer" eggs. There appears to take place two cycles of egg maturing; the first reaches its peak about the middle of July, the second, I am informed, in late August or early September.

It would be of much interest to determine the exact physiologic condition of the eggs and of the sperm at the moment of natural shedding, throughout the breeding season. Vernon's observations ('99) need to be checked by more refined quantitative methods.

Between the first half of July and late August there appears to be a period during which the mature eggs deteriorate rapidly, and few if any unripe eggs mature. The behavior of the eggs in this latter period depends upon the relative numbers of unripe, ripe, and overripe eggs and the degree of overripeness. As these factors seem not to have been taken into account (Gemmill, '00) much confusion has resulted.

The agglutination test is in accord with other tests, all of which force one to conclude that "ovary" eggs show all gradations to extremely overripe eggs.

Other Sources of Variability.

When a comparison was made between the eggs tested immediately after receiving the *Arbacia* from the collecting boat, and eggs from the same group of *Arbacia* kept in tanks of running sea water 3 or more days, the eggs from the latter were more deteriorated. Even when the *Arbacia* were kept several days in the large floats at the wharf in the harbor, the eggs were more deteriorated than those eggs freshly tested upon receipt from the collecting boats. When the *Arbacia* were exposed to the sun during the trip to the laboratory, *i.e.*, when large numbers were kept in pails with insufficient sea water or exposed to the sun, such *Arbacia* gave a preponderance of deteriorated eggs.

When individual *Arbacia* were kept in jars containing 1,000 cc. to two gallons of sea water, changed twice daily, and the jars

placed in running sea water, the germ cells spontaneously shed from the intact animals. These germ cells were tested. The tests were size, color, shape of egg, rate of membrane formation, rate of cleavage and agglutination time. Many such spontaneously shed germ cells were in excellent physiologic condition. *Yet not infrequently such eggs showed surprising degrees of degeneration.* Such degenerate eggs were shed late, while the less degenerate eggs were shed early in the egg cycle. I attribute such degeneration to delayed shedding of eggs.

It was also noted that for several days after severe and protracted storms, *Arbacia* freshly collected and freshly tested gave germ cells in a deteriorated condition. It would seem as though severe storms or other adverse condition delays the natural shedding of germ cells with a consequent degeneration, the extent of degeneration being a function of the time that ripe eggs are retained within the body, and a function of the temperature of the sea water.

Extrusion of eggs, whether spontaneously or after opening the body, gives no assurance that the eggs are recently matured, *i.e.*, in good condition.

My evidence for "shed" versus "testes" sperm is not sufficient to draw any definite conclusions.

Summary.

1. *Arbacia* were freshly collected, freshly opened, the "best" eggs selected and immediately tested for agglutination time. The technique gave for duplicate tests, or for tests of aliquot portions of eggs, a difference in agglutination time of 0 to 4 seconds with an average difference of 1 second or 4.5 per cent. This is the experimental error.

2. (*a*) Eggs from different females when tested separately, under strictly comparable conditions, by the same sperm suspension from a single male, gave extremely wide differences in agglutination time, namely, 2 to 55 seconds or 9 to 1,300 per cent. Eggs that gave high agglutination values with one sperm gave consistently high, though not the same values with sperm from other males.

(*b*) These variations in agglutination time corresponded with

the variations in size, color and shape of eggs, loss of jelly, rate and per cent. of membrane formation, rate and per cent. of cleavage. All of them measure degrees of deterioration or overripening prior to shedding. Hence agglutination time may be used as another quantitative measure of deterioration of eggs.

(*c*) "Shed" eggs gave lower agglutination values and are less variable than "ovary" eggs, as defined, due to larger number of overripe eggs in the "ovary eggs."

(*d*) Severe storms and other adverse conditions that delay spontaneous shedding tend to deteriorate the eggs within the body, with corresponding changes in agglutination values.

3. (*a*) Suspensions of sperm from different males, in the same concentration, tested with the same egg water also gave a surprising amount of variation. They varied from 11 to 3,300 per cent.

(*b*) Sperm which gave high agglutination values with the eggs of one female gave consistently high, though not the same, values with eggs of other females. Sperm with low agglutination values gave low values with other females.

4. These large differences in agglutinability of different freshly shed sperms are due to corresponding physiologic deterioration or overripening prior to shedding.

5. The large differences in freshly shed eggs from different females is in small part due to genetic differences in agglutinin production, in largest part to deterioration of eggs within the body, prior to shedding.

6. Chronologically fresh, *i.e.*, "normal" germ cells may range from physiologically fresh to extremely overripe germ cells.

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CHANGES IN AGGLUTINATION OF AGEING GERM CELLS.

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Previous studies (Goldforb, '17*a*, '17*b*, '18*a*, '18*b*, '29) led to the conclusion that freshly shed eggs, from freshly collected individuals, vary widely in a number of morphologic and physiologic traits, such as size, color, shape of eggs, amount of jelly, duration of agglutination, rate and manner of membrane formation, rate and percentage of segmentation, etc. Freshly shed sperm from different males, freshly collected and freshly prepared, were just as variable in their agglutinability, fertilizability, etc.

This wide range of variability, in all these traits, of so-called "normal" germ cells represented to a minor degree genetic differences, and to a major degree differences in physiologic condition. In other words, during the breeding season the freshly shed germ cells may be freshly matured or in varying and marked degrees of overripeness. When the germ cells were aged outside of the body, no new changes occurred, but merely a continuation or intensification of the morphologic and physiologic changes begun within the body of the sea urchin.

The present study [1] was undertaken to throw further light on the ageing phenomena with particular reference to agglutination, and to correlate the agglutination changes with those previously studied in ageing germ cells.

TECHNIQUE.

For a description of the technique and for evidence of its adequacy I refer to the previous study (Goldforb, '29). It is important to note that the *Arbacia* were freshly collected, the germ cells freshly shed, the eggs washed in 300 cc. of "sterile"

[1] The experiments were performed at the Marine Biological Laboratory at Woods Hole in 1924 and 1926. I wish to acknowledge my thanks to the Directors for the facilities of the Laboratory.

sea water collected at high tide. The ratio of eggs to sea water was usually 1 to 3.[1] The exact ratios are given in the Tables. At the end of one hour a sample of the supernatant egg water was taken, and, if any eggs or jelly were present, they were immediately removed. The egg water was then diluted, and this solution used throughout the experiment. At each age, thereafter, the same procedure was used with the eggs of each female. For each test a 1 per cent. sperm suspension was prepared from a freshly opened male. The results are therefore strictly comparable. The experimental error was 1 second or 4.5 per cent. (Goldforb, '29).

I.

Agglutination When Eggs Only Were Aged.

Eggs in Good Condition When Shed.

This section includes experiments in which ageing eggs were tested, at each successive interval, with freshly prepared 1 per cent. suspensions of freshly shed dry sperm. The results are brought together in Tables II*A* and II*B*. These Tables give the age, or time after shedding of the eggs and sperm, the egg to water ratio at each age for each female, the agglutination in seconds, the difference in agglutination time between the initial and each subsequent age, the calculated difference in agglutination time, when the egg to water ratios were not the same.[2] The tests were made in different dilutions of egg water, but only one is given in the Tables. The other dilutions gave similar results. Fifty-nine tests are recorded.

The results are summarized in Table I; the individual experiments are given in Table II.

[1] Even when egg to water ratios were the same, and the solutions, made at successive intervals, were in the same concentration, the resulting agglutinin concentration was not necessarily the same. For as eggs become increasingly overripe they lose their jelly layer with corresponding increase of egg mass to water. This alteration of the ratio is in part compensated by progressive enlargement of overripening eggs. There appears to be no feasible way of calculating these changes. Fortunately the differences in agglutinin production and reactibility of sperm, due to ageing, are so much greater than the error here indicated that the agglutination phenomenon is not seriously affected.

[2] These calculated values are approximate. It should however be noted that these approximations are in very close agreement with the results obtained when the egg to water ratios were the same at successive ages.

It should be recalled that when the eggs were in good physiologic condition at the time of shedding, and aged at approximately 20° C., under the described conditions, there is no disintegration during the first 24 hours. After 24, and usually after 36 or 48 hours, disintegration may begin with the liberation

TABLE I.

SUMMARY SHOWING CHANGE IN AGGLUTINATION TIME WITH AGEING OF EGGS.

A. Total—All Experiments Combined.

Age of Eggs.	No. of Experiments in Which Agglutination			Per cent. Experiments in Which Agglutination		
	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	1	8	4	8	61	33
4–9 hrs.	7	14	1	32	63	5
22–25 hrs.	9	11	4	37	46	16
B. Eggs in Good Condition when Shed.						
3 hrs.	1	6	3	10	60	30
4–8 hrs.	0	12	1	0	92	7
24–25 hrs.	3	9	2	21	64	14
C. Eggs in Poor Condition when Shed.						
3 hrs.	0	2	1	0	66	33
4–9 hrs.	7	2	0	78	22	0
22–24 hrs.	6	2	2	60	20	20

of anti-agglutinins with a corresponding reduction in agglutination values. Any decrease in values during the first 24 hours may not be attributed to disintegration but to decreased liberation of agglutinins. On the other hand, eggs in poor condition when shed, or eggs in good condition but kept in an adverse environment, such as high temperature, may disintegrate during the first 24 hours. The decreased agglutination values of such eggs may be attributed to lowered agglutinin liberation and to the liberation of anti-agglutinins. The exact physiologic condition of the eggs at the time of shedding is determined by the change in size, color, shape, viscosity, amount of jelly, rate and per cent. of fertilization, regularity and per cent. of cleavage, etc. These symptoms showed that the *eggs in Experiments 5,*

TABLE II A.

SHOWS PROGRESSIVE AND MARKED INCREASE IN AGGLUTINATION TIME WITH AGEING OF EGGS. *Eggs in Good Condition when Shed.* EGG WATER DILUTIONS THE SAME THROUGHOUT EACH EXPERIMENT.

TABLE II *A*.

SHOWS PROGRESSIVE AND MARKED INCREASE IN AGGLUTINATION TIME WITH AGEING OF EGGS. *Eggs in Good Condition when Shed.* EGG WATER DILUTIONS THE SAME THROUGHOUT EACH EXPERIMENT.

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Observed Change in Sec.	Calc. Value in Sec.	Calc. Change in Sec.	Aver. Change in %.
		Eggs.	Sperm.			Eggs.	Sperm.						
9	1	1 hr.	1 hr.	17	1 : 3	24	1	24	1 : 3	+ 7			+ 50
	2			13	1 : 3			23	1 : 3	+ 10			
	3			22	1 : 3			31	1 : 3	+ 9			
12	1	1	1	12	1 : 6	25	1	12	1 : 8		13	+ 1	62
	2			13	1 : 6			18	1 : 8		19	+ 6	
	3			11	1 : 6			15	1 : 17		30	+ 19	
	4			15	1 : 4			7	1 : 30		21	+ 6	
23	1	1	1	19	1 : 2	3	1	54	1 : 2	+ 35			+ 211
	2			18	1 : 2			75	1 : 2	+ 57			
	3			19	1 : 3			46	1 : 3	+ 27			
						5	1	19	1 : 4		29	+ 10	+ 84
								38	1 : 2	+ 20			
								36	1 : 3	+ 17			
						8	1	27	1 : 7		54	+ 35	+ 132
								24	1 : 5		46	+ 28	
								20	1 : 6		30	+ 11	
						24	1	10	1 : 6		20	+ 1	+ 48
								18	1 : 6		36	+ 18	
								10	1 : 6		15	− 5	
24	1	1	1	17	1 : 3	3	1	16	1 : 4		20	+ 3	+ 5
	2			29	1 : 3			24	1 : 4		34	+ 5	
	3			28	1 : 3			18	1 : 5		24	− 4	
						5	1	17	1 : 6		25	+ 8	+ 56
								30	1 : 6		45	+ 16	
								27	1 : 7		46	+ 26	
25	1	1	1	15	1 : 3	3	1	16	1 : 3	+ 1			+ 22
	2			16	1 : 3			29	1 : 3	+ 13			
	3			22	1 : 4			22	1 : 4	0			
	4			16	1 : 3			16	1 : 3	0			
						6	1	55	1 : 4		60	+ 45	+ 103
								24	1 : 3	+ 8			
								24	1 : 3		22	0	
								34	1 : 3	+ 18			
						24	1	0	1 : 6			− 15	− 4
								14	1 : 5		17	+ 3	
								15	1 : 4	− 7			
								16	1 : 7		32	+ 16	

TABLE II *B*.

SHOWS CHANGE IN AGGLUTINATION TIME WHEN THE *Eggs Were in Poor Condition when Shed.*

Exp. No.	♀ No.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Age of		Aggl. in Sec.	Egg to Water Ratio.	Observed Change in Sec.	Calc. Value in Sec.	Calc. Change in Sec.	Aver. Change in %.
		Eggs.	Sperm.			Eggs.	Sperm.						
5	1	1 hr.	1 hr.	17	1 : 4	22	1	17	1 : 5		19	+ 2	
	2			22	1 : 3			13	1 : 5		18	− 4	
	3			13	1 : 4			14	1 : 10		28	+ 15	− 16
	4			26	1 : 1			0	1 : 4		0	− 26	
20A	1	1	1	29	1 : 2	24	1	21	1 : 2	− 8			
	2			21	1 : 2			20	1 : 3		22	+ 1	
	3			24	1 : 3			26	1 : 5		36	+ 12	+ 6
	4			27	1 : 3			26	1 : 4		28	+ 1	
20B	1	1	1	23	1 : 5	4	0	26	1 : 6		29	+ 6	
	2			27	1 : 3			19	1 : 4		21	− 6	0
						6½	0	18	1 : 6		20	− 3	
								12	1 : 4		14	− 13	− 32
						9	0	22	1 : 5		17	− 6	
								19	1 : 4		21	− 6	− 24
						23	0	20	1 : 5	− 3			
								14	1 : 4		16	− 11	− 28
22	1	1	1	21	1 : 3	3	0	21	1 : 3	0			
	2			26	1 : 3			38	1 : 3	+ 12			
	3			18	1 : 3			23	1 : 3	+ 5			+ 21
						5		11	1 : 8		28	+ 7	
								13	1 : 5		20	− 6	− 6
								9	1 : 5		13	− 5	

20A, 20B, and 22 were in poor physiologic condition when shed, and it is precisely in these experiments that little or no increase in agglutination values occurred with ageing of eggs. On the other hand the eggs in Experiments 9, 12, 23, 24, and 25 were, by these symptoms, in good condition when shed, and *it is in these eggs that a definite, progressive and marked increase in agglutination values took place with ageing of eggs.*

In Table I these two groups are separated. Such separation brings out in sharp relief that the *behavior of ageing eggs depends upon their condition when shed.* Part A includes all experiments. Part B includes the experiments in which the eggs were in good condition when shed. *These eggs showed a material increase in agglutination values in 92 per cent. of the tests.* The increase reached a maximum in 3 to 5 hours. Thereafter there was a slow decrease in values. When 24 hours old, 64 per cent. of the tests were still greater than the initial ones.

On the other hand, eggs in poor condition when shed (Part C) gave increased agglutination values with age, in only 66 per cent. of the tests. The maximum values were reached earlier, *i.e.*, in 0 to 3 hours, and are only slightly above the initial tests or no higher. In 22 to 24 hours 60 per cent. of the agglutination values were below and only 20 per cent. above the initial values. See Figures 1*a* and 1*b*.

The analysis of a few experiments will clarify the results and the conclusions.

In Experiment 23, Table II*A*, the eggs were in good condition at time of shedding. Three females were used. Their eggs were tested separately, at each age, *viz.*, 1, 3, 5, 8, and 24 hours, by freshly shed, freshly prepared sperm suspensions. The initial tests gave agglutination values of 19, 18, and 19 seconds respectively. When 3 hours old, and the egg water dilutions the same, the agglutination values were surprisingly greater, namely, 54, 75, and 46 seconds. *The increase in agglutination values was therefore 35, 57, and 27 seconds, or 284 per cent., 416 per cent., and 242 per cent. respectively.* When these eggs were 5 and 8 hours old the values decreased, yet were much greater than the initial values. The 5 hours old eggs agglutinated 10, 20, and 17 seconds longer than the initial tests. The 8 hour old eggs

gave 35, 28, and 11 seconds longer agglutinations than the initial tests. When the eggs were 24 hours old the values dropped sharply; female No. 1 had returned to the initial value, female No. 3 had decreased below, and only female No. 2 was still appreciably above the initial value. In this experiment ageing of eggs gave rise to a very large increase in agglutination. The maximum values occurred about the third hour of ageing, thereafter there was a very slow reduction in values, approximating the initial ones about the twenty-fourth hour.

The results in other experiments are in fundamental agreement with the one just described. The rate and the amount of increase depended primarily upon the degree of overripeness of the eggs at the time of shedding.

In Experiment 25, when the eggs were 3 hours old, only one female increased considerably above initial values, namely 13 seconds. The other 3 females exhibited no change at this age. But when 6 hours old, three of the females gave substantial increases, namely 45, 8, and 18 seconds, respectively, or an average increase of 103 per cent. One female only gave no increase in agglutination values, and this female gave no increase at any age. Reference to my protocol discloses the fact that the eggs of this female were "ovary" eggs, few in number (only 1 cc.)' and by various tests were overripe when shed. These were the only eggs that did not give rise to marked increase in agglutination values with age. With further ageing there was a return towards or below the initial values in all four females. When 24 hours old, two still registered 3 and 16 seconds above, one registered 15 seconds below and one 7 seconds below the initial values.

In Experiment 24, when the eggs were 3 hours old, the change in agglutination was small, namely + 3, + 5, − 4 seconds. When 5 hours old there was a substantial increase in all females, namely + 8, + 16, + 26 seconds respectively, or an average of + 56 per cent.

In Experiments 9 and 12, tests were made when the eggs were one hour and 24 hours old. Intermediate values were not determined. Six out of the 7 females tested gave increased agglutination values *even at the twenty-fourth hour of ageing*. The

increases were 7, 9, and 10 seconds for the 3 females of Experiment 9, and 1, 6, 6, 19 seconds for the 4 females of Experiment 10. The average increase was 56 per cent.

Where differences in egg to water ratios necessitated corrections, the results are in substantial agreement with those experiments in which the egg to water ratios were the same throughout the experiment.

Different males were used at each test with the possibility of introducing thereby a source of considerable variation (Goldforb, '29). The results, however, are surprisingly consistent. *Eggs in good condition when shed gave rise to marked and progressive increase in agglutination values during the first 3 to 6 hours of ageing.* After this age there was a slow reduction in values. By the 24th hour some have been reduced to the initial values, some have not yet done so and a few are below initial values.

It is concluded that eggs in good condition liberate increasing quantities of agglutinin during the first 3 to 6 hours, and liberate decreasing quantities thereafter.

Eggs in Poor Condition When Shed.

The behavior of eggs in poor condition when shed is very different. See Table II*B*. In Experiment 20*B*, for example, the agglutination values for the freshly shed eggs were 23 and 27 seconds respectively. When 4 hours old, the eggs of female No. 1 gave a small *increase* of 6 seconds, those of female No. 2 a *decrease* of 6 seconds. When the eggs were 6½ hours old, both females registered values below the initial ones, namely − 3 and − 13 seconds respectively. When 9 and 23 hours old the values were essentially the same as 6½ hour eggs. These eggs, in poor condition when shed, gave with ageing either an exceedingly small and early increase (in one female only), or a progressive decrease. Lillie, F. R., '14, Lillie, F. R., and Just, E. E., '24, Just, E. E., '19, have described this decreasing agglutination phase. The literature does not contain references to the first or increasing agglutination phase, described above.

In Experiment 22, though the eggs were in moderately good condition when shed, they were precociously aged by the high temperature of the laboratory (29° C.) during the greater part

of the experiment. These eggs gave in the initial tests 21, 26, and 18 seconds respectively for the 3 females tested. When the eggs were 3 hours old, there occurred an increase of 0, 12, and 5 seconds respectively, an increase of 26 per cent. When 5 hours old, with rising temperature, only female No. 1 gave a small further increase in agglutination. The other 2 females gave values below the initial ones.

In Experiments 5 and 20*A* the eggs were in poor condition when shed. They were tested when 1 and 22 hours old. The old eggs registered an increase in two females, no change in three females, and decreases of 4, 8 and 26 seconds in the other 3 females. The average decrease was 5 per cent.

Taking all the experiments in Table II*B* together it is seen that when the eggs were overripe at the time of shedding, agglutination increased in only 6 instances, and the increases were 5 to 15 seconds. In 6 other instances there was no change or the change was within the experimental error and in 10 instances there was a decrease of 4 to 26 seconds.

Figure 1*b* is markedly different from Figure 1*a*, which gives the results with eggs ripe when shed, and shows the considerable increase in agglutination. Figure 1*b*, for overripe eggs, shows a small or no increase.

I interpret these results to mean that beginning with maturation of the egg, there is a progressive and marked increase in the production or liberation of agglutinin, reaching maximal values not at maturity but in 3 to 6 hours later. Thereafter there is a slow progressive decrease in agglutinin production.

Since eggs from different females are in different stages of ripeness, the rate of agglutinin liberation, at a given temperature and H-ion concentration, etc., depended upon the degree of overripeness of the eggs at the beginning of the experiment, as well as the time or subsequent ageing. If just matured there will be a rapid and marked increase followed by a slow decrease. If slightly overripe when shed, there will be a smaller and earlier increase. If more overripe when shed, there will be no increase, but only decreasing agglutinin production. An increase in temperature or in OH ions accelerates the rate of ageing and hence the rate of the agglutinin cycle. It appears that the total amount

of agglutinin is fixed, the more agglutinin liberated prior to the experimental period the less agglutinin is available thereafter.

II.

Agglutination When Sperm Only Are Aged.

The experiments brought together in this section are the reverse of those in the preceding one. A given dry sperm is tested, at each successive age, by freshly prepared egg water solution from freshly shed eggs. These experiments should register the change, if any, in the agglutinability of ageing sperm. The results are brought together in Tables III and IV.

Table III summarizes all experiments. It is evident that *just as in the case of ageing eggs, ageing of sperm is associated with a marked and progressive increase in agglutinability.*

Table III.

Showing Change in Agglutinability of Ageing Sperm.

A. All Experiments Combined.

Age of Sperm.	No. of Experiments in Which Agglutination			Per cent. Experiments in Which Agglutination		
	Decreased.	Increased.	No Change.	Decreased.	Increased.	No Change.
3 hrs.	4	6	5	27	40	32
4–9 hrs.	13	10	6	45	34	20
21–28 hrs.	4	29	0	12	88	0
B. Sperm in Good Condition when Shed.						
3–4 hrs.	0	5	3	0	63	37
5–9 hrs.	0	10	4	0	71	28
21–28 hrs.	1	29	0	3	97	0
C. Sperm in Poor Condition when Shed.						
3 hrs.	3	1	2	50	16	33
5–8 hrs.	13	0	2	87	0	13
24 hrs.	3	0	0	100	0	0

Out of 52 tests 40 per cent. gave increased agglutination values when the dry sperm was 3 hours old. Approximately the same

TABLE IV *A*.

CHANGE IN AGGLUTINATION TIME IN AGEING SPERM. *Sperm in Good Condition* WHEN SHED.

Exp. No.	♀ No.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Egg Water Dilution.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Observed Change in Sec.	Calculated Change in Sec.[3]	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
6	1	1 hr.	1 hr.	17	1/80	1 hr.	24 hr.	40	+ 23			
	2			13				28	+ 15		+ 107	
	3			22				40+	+ 18+			
					1/80	1 [1]	24	58	+ 41			
								20	+ 7		+ 204	
								80+	+ 58+			
					1/320	1 [2]	28	28		+ 56		
								13		+ 26	+ 354	
								51		+ 102		
					1/320	1 [2]	6	26		+ 52		
								13		+ 26	+ 123	
								19		+ 38		
20B	1	1	1	23	1/320	1	4	25	+ 2		− 6	20
	2			27				22	− 5			21
					1/320	1	6½	23	0		− 2	16
								25	− 2			18
					1/320	1	9	29	+ 6			26
								25		+ 1	+ 14	23
					1/320	1	23	37	+ 14			33
								39	+ 12		+ 52	28
19	1	1	1	29	1/320	1	26	50	+ 21	+ 26		26
	2			21				30	+ 9	+ 12	+ 76	29
10A	1	1	4	0		1	25	15	+ 15			12
	2			13				16	+ 3		+ 85	13
	3			10				14	+ 4			11

[1] Egg water 24 hours old. [2] Egg water 28 hours old. [3] Calculated for change in egg water dilution.

TABLE IV *A*.—(*Continued*).

Exp. No.	♀ No.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Egg Water Dilution.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Observed Change in Sec.	Calculated Change in Sec.[3]	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
						1[1]	25	15	+ 15			
								16	+ 3		+ 85	
								14	+ 4			
26	1	1	1	13	1/300	1	21	15	+ 2			
	2			15				19	+ 4		+ 31	
	3			10				16	+ 6			
												18
24	1	1	1	17	1/320	1	3	24	+ 7			25
	2			29				28	− 1		+ 10	26
	3			28				30	+ 2			17
						1	5	25	+ 8			33
								32	+ 3		+ 17	23
								30	+ 2			
												11
25[4]	1	1	1[4]	15	1/300	1	3[4]	15	0			20
	2			16				23	+ 7		+ 11	17
	3			22				20	− 2			12
	4			16				19	+ 3			20
					1/300	1	6[4]	75	+ 60			28
								70	+ 54		+ 220	26
								60	+ 38			20
								16	0			28
					1/300	1	24[4]	0				16
								0			− 100	13
								0				0
								0				

[1] Egg water 24 hours old. [2] Egg water 28 hours old. [3] Calculated for change in egg water dilution. [4] Sperm moderately overripe when shed.

TABLE IV *B*.

CHANGE IN AGGLUTINATION TIME IN AGEING SPERM. *Sperm in Poor Condition* WHEN SHED.

Exp. No.	♀ No.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Egg Water Dilution.	Age of Eggs.	Age of Sperm.	Aggl. in Sec.	Observed Change in Sec.	Average Change in %.	Aggl. Time, Same Egg Water but Freshly Shed Sperm.
22	1	1 hr.	1 hr.	21	1/300	1 hr.	3 hr.	12	− 9		19 Temp. 29° C.
	2			26				20	− 6	− 27	18
	3			18				15	− 3		13
					1/300	1	5	17	− 4		
								18	− 8	− 24	
								14	− 4		
					1/300	1	8	13	− 8		
								12	− 14	− 47	
								9	− 9		
23	1	1	1	19	1/300	1	3A	21	+ 2	+ 1	19
	2			18				18	0		17
	3			19				18	− 1		16
					1/300	1	3B	28	+ 9	+ 57 [1]	
								30	+ 12		
								—	—		No record
					1/300	1	5	19	0		
								17	− 1	− 5	
								17	− 2		
					1/300	1	6	14	− 5		
								15	− 3	− 25	
								13	− 6		
					1/300	1	8	12	− 7		
								12	− 6	− 37	
								11	− 8		
					1/300	1	24	0	− 19		
								0	− 18	− 100	
								0	− 19		
					1/300	1	19B	40	+ 21		
								24	+ 6	+ 46 [1]	
								18	− 1		

[1] This sperm not overripe when shed.

per cent. gave increased values when the sperm was 4 to 9 hours old. But 88 per cent. gave increased values when the sperm was 21 to 28 hours old.

The separation of sperm in good physiologic condition at the time of shedding, from those in poor condition, brings out in sharp relief the marked difference in the behavior of these two kinds of sperm. Sperm ripe when shed (Table III*B*), then aged 3 to 4 hours, gave increased agglutination values in 63 per cent. of the tests. The other 37 per cent. registered no change from the initial tests. When the sperm were 5 to 9 hours old, slightly more (71 per cent.) gave increased values. When the sperm were 21 to 28 hours old, practically all of the tests (97 per cent.) gave agglutination values in excess of the initial ones.

On the contrary, sperm overripe when shed (Table III*C*), and then aged for 3 hours, gave increased values in only 16 per cent. of the tests, while 33 per cent. gave no change, and 50 per cent. gave decreased agglutination values. When the sperm was 5 to 8 hours old not a single test gave values greater than the initial ones, and 87 per cent. gave values below the initial ones. When 24 hours old all the tests (100 per cent.) were below the initial values.

In other words sperm ripe when shed agglutinated *increasingly* during the first 24 to 28 hours. How much longer the values might have increased I did not determine. On the other hand, sperm overripe when shed progressively *decreased*. Ninety-seven per cent. of the tests with ripe sperm gave progressively increased values, while 100 per cent. of the tests with overripe sperm gave progressively decreased values.

As the freshly shed eggs, at each interval, were different, and as this introduces an incalculable variation, the eggs of several females were tested at each age. While there is a variation, the increase in agglutination time, for a given batch of eggs, is so much greater than the variation among different batches, that this variant may be ignored.

It should furthermore be recalled that the increase in agglutination time, in these experiments, must be referred to increased agglutinability of sperm and not to increased agglutinin of the eggs.

Sperm in Good Condition When Shed.

Experiment 6 (Table IV*A*) may be taken as an illustration of the behavior of sperm ripe when shed. The freshly shed dry sperm, in freshly prepared 1 per cent. suspension, was tested by freshly shed eggs of 3 females, and agglutinated for 17, 13, and 22 seconds respectively. A second test was made immediately thereafter with samples of the same egg waters, in the same dilution, but with different sperm, kept 24 hours in the dry state.[1] All tests were made with fresh 1 per cent. sperm suspensions. *The 24 hour sperm gave a large and unexpected increase in agglutination values in all 3 females*, namely, 23, 15, and 18 seconds, or 107 per cent. greater than the initial tests. That this large increase was not fortuitous was shown by testing samples of the first egg waters, 24 hours later, with the first male whose sperm was now 24 hours old. The agglutination values were again greatly increased in every test, by 41, 7, and 58 seconds, or 204 per cent. When the sperm was 28 hours old, and tested against samples of the same egg waters, all 3 females registered values greater than when the sperm was 24 hours old. The average increase was now 354 per cent. When the same egg waters were tested by other sperm, 6 hours old, though the increase in values is very large (123 per cent.) it is lower than the 28 or the 24 hour sperm. The increase in agglutination values is correlated with the age of the sperm. While the corrections for differences in egg water dilution are only approximate, there can be no doubt but that old sperm gave consistently and in every test very much greater agglutination values than freshly shed sperm.

In Experiment No. 19, the fresh sperm gave a 29 and a 21 second reaction with the eggs of two females. The next test was made when the dry sperm was 26 hours old, and gave an observed increase of 21 and 9 seconds, a calculated increase of 26 and 12 seconds, or 76 per cent. The same egg waters were immediately tested with a fresh sperm and gave only -3 and $+8$ seconds, or 10 per cent. increase beyond initial values.

Experiments 10*A* and 26 gave similar results. At the end of 21 hours the 3 females in Experiment 26 averaged 31 per cent.

[1] At 20° C.

greater, and in Experiment 10 at the end of 25 hours they averaged 85 per cent. greater than the initial values. Tests with fresh sperm gave essentially initial values in 2 out of 3 tests.

In Experiment 24 the sperm was tested when 1, 3, and 5 hours old. The 3 hour sperm gave a very small increase (10 per cent.), the 5 hour sperm gave a little larger increase (17 per cent.). The maximum values had presumably not been reached at this age. Tests with fresh sperm gave no increase.

In Experiment 20*B* there was little or no increase in values when the sperm was 4 and 6 hours old. When 9 hours old the increase was 14 per cent. When 23 hours old the increase was 52 per cent. At each age, not only was the sperm tested by freshly shed eggs, but these eggs were again tested by freshly shed sperm. Comparison of these tests emphasizes the conclusion that ageing of dry sperm gave rise to progressively increased agglutination values, while non-aged sperm gave close to the initial values. Figure 3*a* represents this rise in agglutination with ageing of sperm.

Experiment 25 is an example of a different type of experiment, in which sperm were moderately overripe when shed. The agglutination values of the freshly shed germ cells were 15, 16, 22, and 16 seconds for the 4 females tested. When the dry sperm was 3 hours old it was tested with egg waters from the freshly shed eggs of 4 other females. The change in values was exceedingly small, 0, + 7, − 2, + 3 seconds respectively. The average increase was only 11 per cent.

When, however, the sperm was 6 hours old (and tested with freshly shed eggs from 4 other females) the agglutination values sharply increased by 60, 54, 38, and 0 seconds beyond the initial ones, or an average increase of 220 per cent. While it was improbable that this large increase might have been due to the difference in agglutinin production of the 2 groups of eggs, this could only be demonstrated by actual tests. This was done. Samples of egg water from the same freshly shed eggs were tested with freshly shed sperm, all dilutions being the same. The agglutination values were only little more than the initial tests, namely 20, 28, 26, 20 seconds respectively. In other words, the difference in agglutinability between the two fresh sperms was

only 5, 12, 4, and 4 seconds, or 36 per cent. These differences are attributable primarily to differences in agglutinin production in the two batches of eggs. The difference in agglutinability of the same sperm when fresh and when 6 hours old was 220 per cent. This marked difference cannot be attributed to difference in the eggs but to the change in the sperm during 6 hours of ageing.

When the dry sperm was 24 hours old, the sperm were no longer agglutinable.[1] The sperm moderately overripe when shed, differed from the ripe sperm in reaching its maximum agglutinability precociously, *i.e.*, about the sixth hour, and in more rapidly decreasing thereafter (Figure III*b*).

Sperm Overripe When Shed.

In other experiments the sperm were very overripe when shed. This was determined by (1) the distinct brown color of the dry sperm instead of the light cream color of ripe sperm; (2) the less viscous condition of the dry sperm in contrast to the very viscous or "dry" condition of ripe sperm; (3) the short viability and (4) the early cessation of movement. The sperm in Experiment 23, the best in the day's collection of sea urchins, was overripe by these tests. In Experiment 22, the sperm originally good was precociously deteriorated by the high temperature that prevailed during the greater part of the experiments, namely, 29° C. In both these experiments there was no evidence of an increase in agglutination values with ageing of the sperm. In both experiments there was a slow, direct and definite lowering of agglutination values with ageing of sperm. In Experiment 23 the sperm of 2 males, namely A and B, were tested separately with the egg waters of three females. The sperm were allowed to age for 3 hours. One sperm, A, was overripe when shed, the other, B, not overripe. The overripe sperm when 3 hours old gave values equal to the initial tests, +1 per cent, the non-overripe sperm gave substantially increased agglutination values, 57 per cent. The overripe sperm was again tested when 5, 6, 8 hours old and gave progressively lower agglutinations, namely, −5, −25, −37, −100 per cent respectively. When 24 hours old no

[1] It should be noted that the eggs produced sufficient agglutinin, for, with ripe fresh sperm, agglutination was 28, 16, 13 and 0 seconds respectively.

agglutination occurred. The ripe sperm, on the other hand, when 19 hours old, gave with the same egg waters very substantial increases in agglutination, namely, 46 per cent.

In Experiment 22 the original tests gave agglutination values of 21, 26, and 18 seconds respectively. When the sperm were aged at a temperature of 29° C. for 3 hours, there was a decrease of −9, −6, and −3 seconds, or a decrease of 27 per cent. When aged 5 hours at this temperature there was no further change. At the end of 8 hours there was a decrease of 47 per cent. See Figure III*c*.

It is evident that differences in the degree of overripening of sperm, when shed, gives rise to marked differences in agglutination, just as in the case of eggs. The behavior in both eggs and sperm is dependent on (1) the degree of overripeness at the time of shedding, (2) the ageing or time after shedding, (3) the physical conditions in which they are aged, such as temperature.

Sperm, ripe when shed, and aged under conditions not too injurious shows a progressive increase in agglutination values. This increase begins about 3 hours after the beginning of the experiment, the time varying with the condition of the sperm when shed. Maximum values were reached about the 24th hour. Ninety-seven per cent. of the tests gave maximal values when sperm was 24 hours old.

On the other hand, sperm overripe when shed, or ripe sperm aged at high temperatures, gave no increase in agglutination values with ageing of the sperm. In only one instance was there a negligible increase of 2 seconds. The others decreased progressively in agglutination values with ageing of sperm.

While overripe sperm decreased continuously, ripe sperm underwent a cyclical rhythm, increasing progressively during the first 28 hours, decreasing progressively thereafter.

Agglutination of Ageing Sperm by Ageing Eggs.

The previous two sections led to the conclusion that ageing of eggs or of sperm markedly and progressively prolonged the agglutination reaction, provided the germ cells were not too overripe when shed. It might be urged that in these experiments there was the unpredictable variation due to the use of different eggs or of different sperm at successive ages.

This source of variation is eliminated when the same eggs and the same sperm are used in the same dilutions at successive ages. The data are presented in Table V.

To interpret the results it is again necessary to separate the experiments in accordance with the condition of the germ cells at the beginning of the experiment. In Group A the eggs and the sperm were both in good physiologic condition. In Groups B and C one or the other germ cell was overripe when shed. In Group D both germ cells were overripe.

Group *A*. Eggs and Sperm Ripe When Shed.

In Experiment 9, the egg to water ratios were the same throughout and hence no corrections need be made. In this experiment the freshly shed germ cells gave 17, 13, and 22 seconds for the three females tested. When the same egg water (now 23 hours old), in the same dilution as in the initial test, was used with the same sperm (now 26 hours old), the agglutination increased by 42, 6, and 44 seconds respectively, or 177 per cent. This increase is due to the changes in the ageing sperm. Further support of this conclusion is given by the experiment in which the same 24 hour old sperm was tested by freshly shed eggs of 3 females. The results were very similar, namely, 184 per cent. greater than the initial test. When equally aged eggs were tested with freshly shed sperm the values were only 50 per cent. greater than the initial tests. This gives the measure of change in ageing eggs. When, however, the same 24 hour eggs were tested with the same 24 hour sperm the values were not only much greater than the initial ones, much greater than when the eggs alone were aged, but as great or greater than when sperm alone were aged, namely 192 per cent.[1] It seems that the large increase in agglutination was determined largely by the ageing of sperm rather than by the eggs, and that aged eggs tested by aged sperm gave higher agglutination values than when eggs or sperm were aged.

In Experiment 10 the freshly shed eggs of four females were tested separately by a suspension of a 4 hour dry sperm. The

[1] The agglutination of female No. 2 is given as 9 seconds. This is probably an error and should read 19 or 29 seconds with corresponding greater average per cent. increase.

TABLE V.

SHOWING CHANGE IN AGGLUTINATION TIME WHEN SAME EGGS AND SPERM ARE TESTED AT SUCCESSIVE AGES.

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
							Group A. Eggs and Sperm Ripe when Shed.						
9*a*	1 hr.	1 hr.	17	13	22		1 : 3, 3, 3	1/80					
j	24	26	59	19	66		1 : 3, 3, 3	1/80					+ 177
d	24	24	82	9 [2]	61		1 : 3, 3, 3	1/80					+ 192
g	24	1	24	23	31		1 : 3, 3, 3	1/80					+ 50
b	1	24	58	20	70		1 : 3, 3, 3	1/80					+ 184
10*o*	1	4	12	13	11	15	1 : 6, 6, 6, 4	1/320					
q	22	25	31	20	22	26	1 : 6, 6, 6, 4	1/320					+ 94
t	22	1	21	13	17	25	1 : 6, 6, 6, 4	1/320					+ 49
r	1	25	15	16	14	17	1 : 3, 3, 5, 8	1/320	11	12	13	21	+ 11
w	22	25 + 1 [1]	18	13	17	20	1 : 6, 6, 6, 4	1/320					+ 33
19*a*	1	1	29	21			1 : 2, 2	1/320					
c	26	26	50	39			1 : 2, 3	1/320		42			+ 84
b	1	26	50	30			1 : 3, 3	1/320	55	33			+ 76
f	26	1	18	20			1 : 2, 3	1/320		22			− 20
24*a*	1	1	17	29	28		1 : 3, 3, 3	1/320					
h	3	3	15	26	20		1 : 4, 5, 5	1/320	17	35	28		+ 8
i	5	5	18	23	27		1 : 6, 6, 7	1/320	27	34	46		+ 44
	1	5	25	32	30		1 : 6, 6, 7	1/320	36	48	51		+ 82
	5	1	17	30	27		1 : 6, 6, 7	1/320	25	45	46		+ 56

TABLE V.—(*Continued*).

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
Group B. Overripe Sperm and Ripe Eggs.													
25a	1	0	15	16	22	16	1 : 3, 3, 4, 3	1/300					
l	3	3	13	18	16	11	1 : 3, 3, 4, 3	1/300					− 16
m	6	6	66	40	38	36	1 : 3, 3, 2, 3	1/300			29		+ 147
o	24	24	0	6	14	24	1 : 6, 5, 4, 7	1/300		8	14	30	− 36
k	1	24	0	0	0	0	1 : 3, 3, 4, 3	1/300					− 100
g	24	1	0	14	15	16	1 : 6, 5, 4, 7	1/300		17		32	− 7
s1	24	3		28			1 : 5	1/300					
s2	24	20		55			1 : 5	1/300					
23a	1	0	19	18	19		1 : 2, 2, 3	1/300					
r	3	3	33	93	34		1 : 2, 2, 3	1/300					+ 185
s	5	5	15	29	27		1 : 4, 3, 3	1/300	22	31	27		+ 43
u	8	8	13	15	12		1 : 7, 5, 6	1/300	26	29	18		+ 30
w	24	24	0	0	0		1 : 6, 6, 6	1/300					− 100
w1	24	20	8	11	0		1 : 6, 6, 6	1/300	16	22			+ 2
w5	1	24	0	0	0								− 100
w4	1	20	0	0	0								− 100
w3	1	18	30	24	14								+ 21
	24	1	10	18	10		1 : 6, 6, 6	1/300	20	36	15		+ 48

TABLE V.—(*Continued*).

Exp. No.	Age of		Aggl. Time in Sec.				Egg Water Ratio.	Egg Water Dilution.	Corrected Values.				Average Change in %.
	Eggs.	Sperm.	♀ 1	♀ 2	♀ 3	♀ 4			♀ 1	♀ 2	♀ 3	♀ 4	
							Group C. Overripe Eggs and Ripe Sperm.						
5*a*	1	1	17	22	13	26	1 : 4, 3, 4, 1	1/80					
b	4	4	13	13	7	12	1 : 8, 8, 8, 4	1/80	19	20	11	24	− 5
c	6	6	26	12	10	31	1 : 4, 4, 4, 2½	1/80	26	13	10	46	+ 17
d	22	22	21	9	8	12	1 : 5, 5, 10, 4	1/80	23	12	17	24	− 2
f	1	22	25	18	11	60	1 : 4, 3, 4, 1	1/80					+ 46
h	22	1	17	13	14	0	1 : 5, 5, 10, 4	1/80	19	18	28		− 16
20*p*	1	1	23	23			1 : 5, 3	1/320					
q	4	4	23	17			1 : 5, 4	1/320		19			− 6
r	6½	6½	26	21			1 : 6, 4	1/320	27	23			0
s	9	9	14	14			1 : 5, 4	1/320		16			− 40
t	23	23	26	24			1 : 5, 4	1/320		27			+ 6
o	1	23	37	39			1 : 5, 4	1/320		42			+ 58
n	1	9	29	25			1 : 5, 4	1/320		28			+ 14
j	23	0	20	14			1 : 5, 4	1/320		16			− 28
							Group D. Overripe Eggs and Overripe Sperm.						
3*a*	1	1	8	7	16		1 : 5, 5, 2	1/120					
b	3	3	8	8	21		1 : 5, 5, 2	1/120					+ 31
c	6	6	11	8	15			1/120					+ 9
g	24	24	0	0	0		1 : 5, 6, 4	1/120					
j	24	1		7	9		1 : 5, 6, 4	1/120		8	13		− 9
f	1	24	0	0	0		1 : 5, 6, 4	1/120					− 100
22*a*	1	1	21	26	18		1 : 3, 3, 3	1/320					
b	3	3	24	29	27		1 : 3, 3, 3	1/320					+ 23 [2]
l	5	5	11	12	14		1 : 8, 5, 5	1/320	19	15	17		− 21 [2]
m	8	8	10	11	9		1 : 10, 6, 5	1/320	20	17	13		− 22 [2]
g	8 [3]	1	17	18	13		1 : 10, 6, 5	1/320	34	27	20		+ 24
j	1	8 [3]	13	12	9			1/320					− 47

[1] Mixture of 25 hr. and 1 hr. sperm.

[2] Probably should read 19 or 29.

[3] Temp. 29° C.

agglutination values were 12, 13, 11, and 15 seconds respectively. The second test was made 21 hours later with the same germ cells. These 22 hour eggs, as in other experiments, were washed just as in the initial test, the supernatant liquid was in the same egg to water ratio for the same time (1 hour), samples of the egg water were diluted to the same concentration, and immediately tested. Suspensions of the dry sperm, now 25 hours old, were freshly made in the same 1 per cent. concentration, with the same stock of "sterile" sea water. Under these comparable conditions the agglutination values were larger in every instance, viz., 19, 7, 11, and 11 seconds respectively, *i.e.*, an average increase of 94 per cent. Other samples of the same egg waters (of these 22 hour eggs) were then tested with freshly shed sperm (Exp. t) and gave longer agglutination values than the initial tests by 49 per cent. This is in accordance with the results already described for ageing eggs. When the 25 hour sperm (Exp. r) was tested with freshly shed eggs the values were only 11 per cent. greater than the initial tests. This is interpreted to mean that the sperm had passed the peak of agglutinability. When, however, these same overripe eggs and sperm were tested together the values were far greater than when the eggs or the sperm were aged alone, namely 94 per cent. greater than the initial tests.

In Experiment 19 the freshly shed germ cells gave a 29 and a 21 second agglutination reaction. When the same eggs, 26 hours old, were tested by freshly shed sperm, the values were 20 per cent. *below* the initial ones, indicating low agglutinin production of the senescent eggs. The sperm were equally aged (26 hours old), yet when tested with freshly shed eggs the values were 76 per cent. above the initial ones. When this overripe sperm was used with the overripe eggs the values were *somewhat larger* than when aged eggs or aged sperm alone were used, for the increase was now 84 per cent. greater than the initial tests.

In Experiment 24 the germ cells were tested when 1, 3, and 5 hours old, only. When both germ cells were 3 hours old there was little change, +8 per cent. When 5 hours old the increase was greater, namely 44 per cent.

It is concluded that old eggs tested with old sperm gave in

every instance values greater than either old eggs or old sperm and much greater than the initial values. The results are plotted in Fig. VII.

Overripe Sperm and Ripe Eggs. Group *B*.

In Experiment 12 the sperm were overripe when shed. When the germ cells were freshly shed the agglutination values were 12, 13, 11, and 15 seconds respectively. When 25 hours old no agglutination occurred in any of the 4 females. The 25 hour sperm was then tested with freshly removed eggs of 4 females. No agglutination occurred in any test, showing that the sperm were excessively overripe and not agglutinable. When, however, the 25 hour eggs, in the same egg water ratio, were tested with freshly removed sperm, the agglutination values were 12, 18, 15, and 7 seconds. This showed that the eggs were still liberating abundant agglutinin, and therefore not responsible for the lack of agglutination in the previous tests with old sperm. The old sperm had deteriorated precociously, due to the aged condition at the beginning of the experiment.

In Experiment 25 the sperm were only moderately overripe when shed. Tests were made at 1, 3, 6, and 24 hours. The 3 hour germ cells gave somewhat lower values than the initial ones, viz., −16 per cent. When 6 hours old there was a marked increase of 147 per cent. When the eggs and the sperm were 24 hours old, the agglutination values dropped sharply to a level below the initial ones, namely −36 per cent. Part of this sharp drop is due to the fact that the eggs of female No. 1 were senescent and liberated no agglutinin. The other 3 females liberated nearly as much agglutinin as in the initial test. The old sperm were also quite senescent. For, when tested with the freshly shed eggs of 4 females, they did not agglutinate in a single instance. Yet when these senescent sperm were used with old but not senescent eggs, they agglutinated 0, 8, 14, and 30 seconds respectively.

The precocious senescence of the sperm was due to the overripe condition at the time of shedding and to subsequent ageing. Such senescent sperm attain maximal agglutination values early, not when 24 hours old, as in experiments with ripe germ cells, but when 6 hours old.

It should also be noted that when the 24 hour eggs were tested with 1 hour sperm the agglutination lasted 17 seconds. When 3 hour sperm was used the agglutination lasted 28 seconds. When 20 hour sperm was used the agglutination lasted 55 seconds. When, however, 24 hour sperm was used no agglutination occurred. Agglutination had increased with ageing of sperm, until the maximum was reached, about 20 hours after shedding. Then followed a very rapid decrease, so that 4 hours later no agglutination occurred.

In Experiment 23 the sperm were overripe when shed. The results are in substantial agreement with the previous experiment. Maximal values occurred precociously, *i.e.*, when the germ cells were only 3 hours old. The increase averaged 185 per cent. When 5 hours old the agglutination values were only 43 per cent. above initial ones, when 8 hours old, 30 per cent. above, and, when 24 hours old no agglutination occurred in any of the tests. Further tests showed (Exp. W^3, W^4, W^5) that the sperm had suddenly become non-agglutinable between the 18th and the 20th hour. The eggs were in moderately good physiologic condition when 24 hours old, giving an average of 48 per cent. above initial values, but the sperm were quite senescent even when 20 hours old. Yet these senescent sperm were not only agglutinated by these old eggs but agglutinated for almost as long periods as in the initial tests.

The results are plotted in Fig. 7*b*.

Some change has taken place in addition to the increase in agglutinin of ageing eggs. For the increase in agglutination is greater than can be accounted for by the agglutinin alone. The additional factor or group of factors not only lengthens the agglutinating time but causes sperm to agglutinate after they have ceased to do so with freshly shed eggs. This factor occurs concomitantly with ageing of sperm.

Overripe Eggs and Ripe Sperm. Group *C*.

When the eggs were overripe but the sperm not overripe at the time of shedding, the resulting behavior is different.

In Experiment 5 the eggs of the 4 females were overripe when shed. The sperm were ripe. These germ cells gave initial values of 17, 22, 13, and 26 seconds respectively. When 4 hours

old the values were practically the same, *i.e.*, −5 per cent. When 6 hours old there was a small increase, *i.e.*, 17 per cent. When 22 hours old the values had returned to the initial ones, −2 per cent. When the germ cells were tested separately, the old eggs with freshly shed sperm averaged 16 per cent. below the initial values. This low average was in part due to the entire lack of agglutinin production by female No. 4, and in part due to the low agglutinin production of the other senescent eggs. On the other hand the 22 hour old sperm tested by freshly shed eggs gave 46 per cent. higher values than in the initial tests. This showed that the sperm were not senescent. The lack of agglutination in the previous 22 hour test was clearly due to insufficient agglutinin and hence the real agglutinability of the sperm could not be evidenced.

The eggs in Experiment 20 were also in poor condition when shed. These eggs gave at the end of 4 hours essentially the same as the initial values, *i.e.*, −6 per cent. When 6½ hours old there was practically no change, 0 per cent. When 9 hours old there was a decrease of −40 per cent. But when 23 hours old there was a return to initial values, *i.e.*, +6 per cent. The explanation is found in testing the germ cells separately. The 9 hour sperm tested with freshly shed eggs gave an increase of 14 per cent. The 23 hour sperm gave an increase of 58 per cent. over initial values. On the other hand the 23 hour eggs, tested by freshly shed sperm, gave a decrease of −18 per cent. The low values for these old eggs indicate the degree of their senescence. The high values for the old sperm indicate corresponding lack of senescence. When such senescent eggs are tested by old but non-senescent sperm, the values are not increased as was the case when old but non-senescent eggs were used. In other words *sufficient agglutinin is necessary for the sperm to manifest the marked and progressive increase in agglutinability with over-ripening.*

It appears that while agglutinin of eggs decreased with ageing, the agglutinability of the sperm was markedly increased. The resultant values are the summation of the amount of agglutinin given off by the eggs and of the agglutinability of the sperm at the time of the experiment.

When eggs and sperm were both ripe when shed, and then tested at successive ages, agglutination increased at first because agglutinin of eggs increased. Later the increase in agglutination was due to the late increase in agglutinability of sperm. Maximal increases are high and late. When the eggs were overripe and the sperm ripe at the time of shedding, maximal agglutination was low and early, due to progressively lowered agglutinin production. When sperm were overripe and eggs ripe at the time of shedding, maximal agglutination occurred early, but the values were high. This is due to adequate agglutinin but lowered agglutinability of the sperm.

Overripe Eggs and Overripe Sperm. Group *D*.

When eggs and sperm were both overripe when shed (Exp. 3), or, when the germ cells were ripe but precociously aged by high temperature (29° C.), Experiment 22, there was with ageing a smaller and earlier increase in agglutination.

In Experiment 3, when the germ cells were 3 hours old, the values were 31 per cent. above the initial tests; when 6 hours old, only 9 per cent. above initial values; and when 24 hours, no agglutination occurred. The rapid decrease is apparently due to early senescence of the sperm. The 24 hour sperm were clearly senescent for they did not agglutinate with freshly shed eggs. Similarly, in Experiment 22, agglutination increased +23 per cent. when the germ cells were 3 hours old, decreased to −21 per cent. when 5 hours old, and to −23 per cent. when 8 hours old.

The explanation is due, as before, to precocious reduction of agglutinability of the sperm and to precocious reduction in agglutinin production of eggs.

Reference to Figs. 1 to 7 will bring out sharply the differences in behavior due to the degree of overripeness of each of the germ cells. The ordinates represent change in agglutination time, in per cent. as compared with the initial tests. The abscissas represent age of germ cells in hours.

In other words, in order that the increasing phase in the agglutination phenomenon be manifested, it is necessary that both the germ cells be not too overripe at the time of shedding.

The evidence demonstrates the cyclical change in agglutinin production by ageing eggs, (2) the cyclical change in agglutinability of ageing sperm. (3) When both eggs and sperm aged,

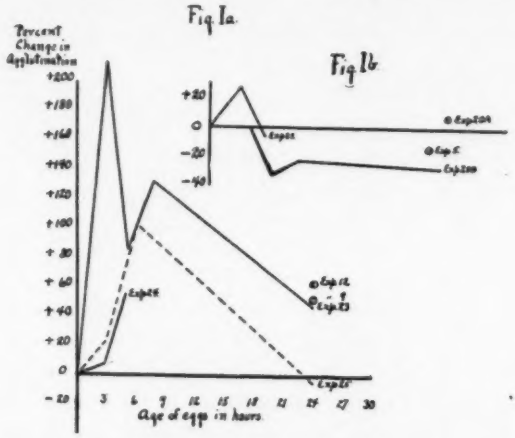

FIG. 1a. The agglutination cycle when eggs alone were aged, and when such eggs were in good physiologic condition at the time of shedding. Each graph is the average for all females of a series. These graphs show the marked and progressive increase and subsequent decline in values.

FIG. 1b. The agglutination cycle for ageing eggs when the eggs were overripe at the time of shedding. There is a small or no initial increase, and a slow decrease in values thereafter.

agglutination is a resultant of the amount of agglutinin produced by the eggs at a given age, and the degree of agglutinability of the sperm at that age.

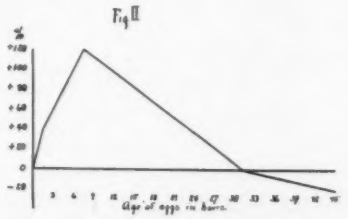

FIG. 2. The probable average agglutination cycle of ageing eggs from maturation to death, showing the marked progressive and early increase in agglutinin production.

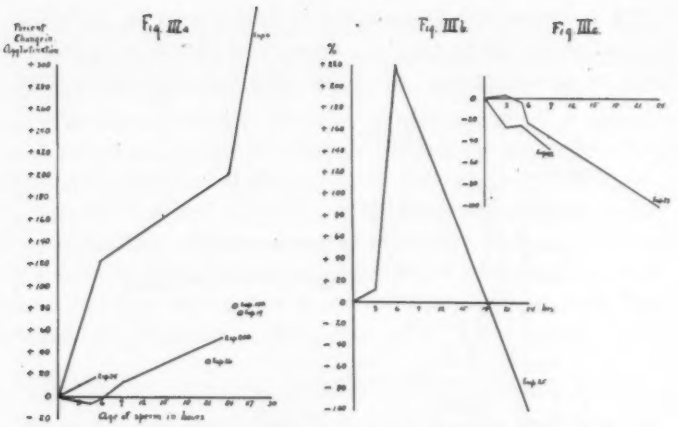

FIG. 3*a*. The agglutination cycle when sperm alone are aged and when the sperm are ripe at the time of shedding. This figure shows the marked, progressive, but slower increase in agglutinability with age.

FIG. 3*b*. The curve for ageing sperm, when the sperm is moderately overripe when shed, showing the precocious and large increase in agglutinability, and the very rapid decrease thereafter.

FIG. 3*c*. The agglutination cycle for ageing sperm, when the sperm were more overripe at the time of shedding. This shows a progressive decrease only.

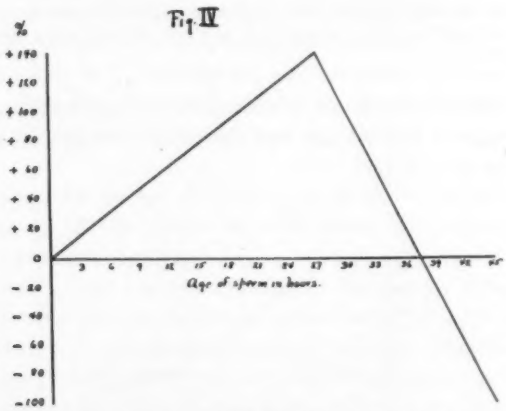

FIG. 4. The probable average agglutination cycle for the agglutinability of the sperm from maturation to death. The sperm cycle attains its maximum more slowly and decreases thereafter more quickly than the egg cycle.

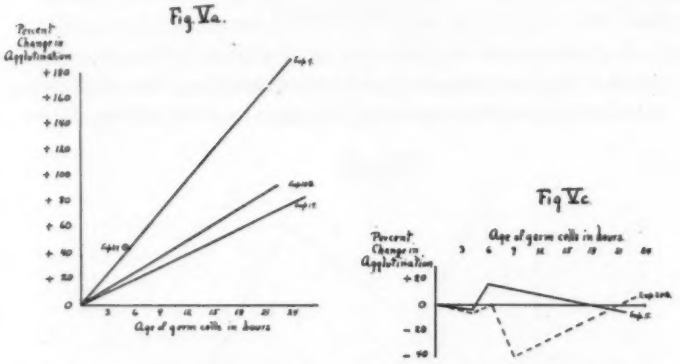
Fig. Va.
Percent Change in Agglutination
Age of germ cells in hours
Fig. Vc.
Percent Change in Agglutination
Age of germ cells in hours

FIG. 5*a*. The agglutination changes when both the germ cells are ripe at the time of shedding and the same germ cells used at successive ages. The graphs strongly suggest the large influence of the sperm. Note the marked increase in agglutination.

FIG. 5*c*. When eggs were overripe at the time of shedding no marked nor progressive increase occurred, due to insufficient agglutinin.

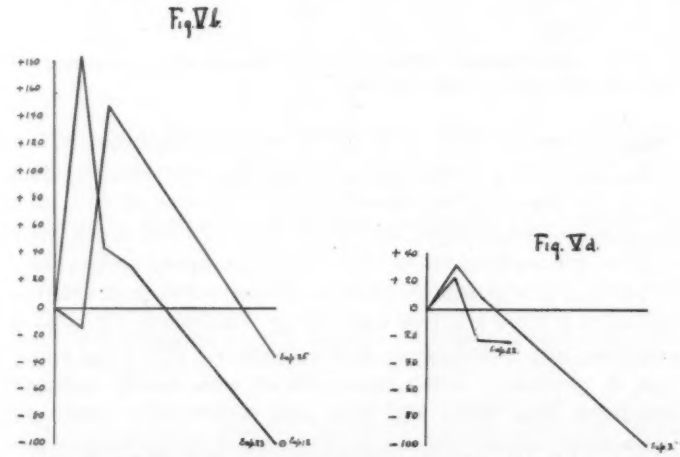

FIG. 5*b*. When the sperm were overripe at the time of shedding the maximum is reached earlier, 3 to 6 hours instead of 24–28 hours; the decline thereafter is correspondingly more rapid.

FIG. 5*d*. When the sperm and eggs were overripe at the time of shedding there is a small and early increase, due to insufficient agglutinin and low ability of sperm to react.

Discussion.

To understand the behavior of the germ cells one must remember that they are matured before shedding, that the interval between maturation and shedding may be brief or may be very

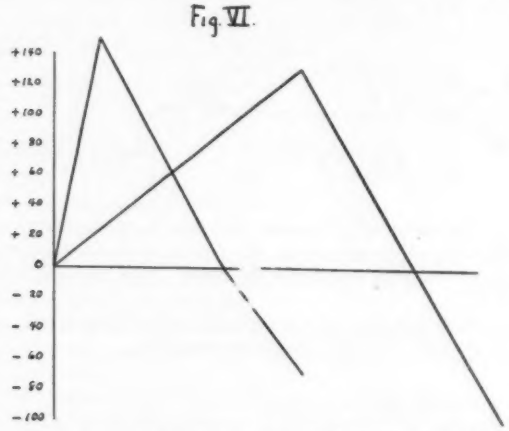

Fig. 6. Gives schematically the trend, when both germ cells were ripe (left) and when partially overripe (right) when shed.

long, that storms and other factors extend this period (Goldforb, '29). Within the body overripening progressively takes place, the degree of overripening being a function of time since maturation, temperature and H-ion concentration of the body fluid or surrounding sea water. I wish to stress the fact that, in addition to a genetic variation in the germ cells from different individuals, there is a very much larger variation at the time of shedding, due to the extent of overripeness. This large variation in physiologic condition occurs even in freshly collected specimens from which the germ cells were freshly shed and immediately tested. One finds all degrees of change from underripe to extremely overripe germ cells. This large variability in "normal" germ cells occurs at the height of the breeding season (Goldforb, '29). Whether "ovary" or "shed" eggs are used seems not to be important. The degree of overripeness is however of the utmost importance.

These differences in physiologic age give rise to corresponding differences in behavior before and after fertilization and during development. It is therefore necessary in comparing germ cells from different individuals to choose by suitable tests those which are in nearly the same stage of ripeness or overripeness.

Fig VII.

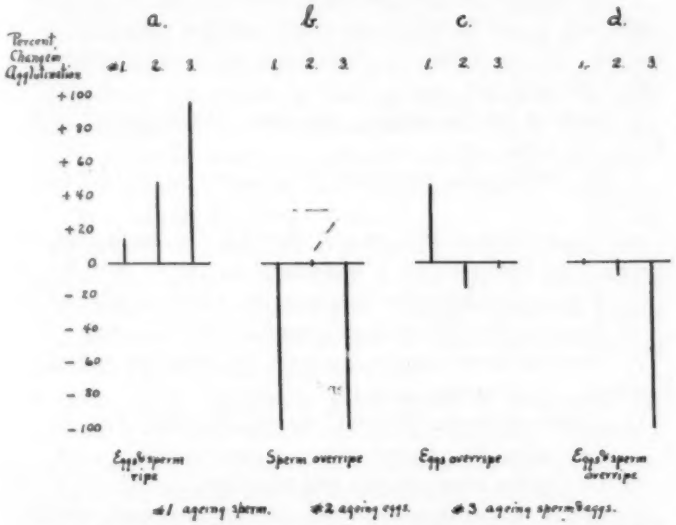

FIG. 7*a*. A typical experiment in which the sperm alone, No. 1, eggs alone, No. 2, and both, No. 3, were aged 24 hours. In the last instance the agglutination is much greater than either of the other two. Both germ cells were ripe when shed.

FIG. 7*b*. A typical experiment in which the sperm alone were overripe when shed. When 24 hours old this sperm did not agglutinate with either fresh eggs, No. 1, or with old eggs, No. 3. The old eggs produced almost as much agglutinin as in the initial test, No. 2. The total lack of agglutination was not due to absence of agglutinin but to inability of sperm to agglutinate.

FIG. 7*c*. The eggs were overripe when shed. The old sperm agglutinate well, No. 1, the old eggs produce little agglutinin, No. 2. This prevents the old sperm from manifesting its increased agglutinability, No. 3.

FIG. 7*d*. Sperm and eggs were both senescent when shed. Total lack of agglutination is due to low agglutinin and to low agglutinability of the sperm.

The changes which occur with overripening are the same within the body and outside the body. These changes may be summarized as follows:

A. 1. Size of eggs increases with overripeness.

2. Color of eggs fade.

3. Shape of eggs becomes less globular, more ellipsoid.

4. Viscosity decreases (Heilbrunn, '15, '26).

5. Jelly layer decreases.

6. Fusion of eggs increases (Morgan, '95, '24, Driesch, '00, Goldforb, '13, deHaan, '13, Goldforb, '18).

7. Fertilization membrane forms closer to egg, is more scalloped and is not found in extremely overripe eggs (Loeb, '03, Harvey, '10, '14, Lillie, '14, Heilbrunn, '15, Goldforb, '17, '18*a*, '18*b*, Lillie and Just, '24).

8. Artificial parthenogenesis increases (Matthews, '01, Lillie, R. S., '14, Loeb, '03).

9. Self fertilization increases (Morgan, '05, '10, '24, Fuchs, '14, '15).

10. Cross fertilization increases (Hertwig, O., and R., '86, '87, Vernon, '00, Tennant, '10, Kupelweiser, '09, '12).

11. Polyspermy increases (Hertwig, '85, Lillie, F. R., '19).

12. Segmentation increasingly irregular (Hertwig, '85).

13. Developmental energy decreases (Vernon, '99, Lillie, '14, Goldforb, '18, Newman, '21).

14. Change in larvæ (Tennent, '10, '11, Koehler, '15).

15. Dry sperm less creamy, more tan, less viscous.

16. Dry sperm loses mobility and viability.

17. Sperm suspensions decrease in mobility and viability (Gemmill, '00, Lillie, '14).

18. Sperm suspensions decrease in metabolism (Cohn, '18).

B. 1. Increasing or more rapid fertilizability, then decreasing or slower fertilizability (Gemmill, '00, Morgan, '04, '05, '10, Cohn, '18, Goldforb, '18*a*, '18*b*, Paspaleff, '27).

2. Parthenogenesis increases, then decreases (Lillie, R. S., '08, '15, Herlant, '18, '19, Loeb, '03).

3. Rate of fertilization membrane formation increases, then decreases (Morgan, '04, '05, '10).

4. Rate of segmentation increases, then decreases (Fuchs, '14, Goldforb, '18*a*, '18*b*).

5. Cleavage irregular then regular (Gemmill, '00).

6. Egg improves, then deteriorates (Lillie, R. S., '08, '15).

7. Agglutinin formation by eggs increases, then decreases (Goldforb, '29, Hinrichs, '27).

8. Agglutinability of sperm increases, then decreases with further overripening.

The *A* group of changes represents the phase stressed by previous workers, namely a progressive change in one direction with overripening.

The *B* group represents a cyclical change, including a prior or vitalizing phase, followed by a decreasing or senescent phase. The symptoms of physiologic change in Group *B* strongly indicate that germ cells are not at their optimum, whether for fertilization, agglutination or segmentation, when they have just matured. A certain degree of ageing or overripening is necessary for optimal results. Beyond this optimum there is a return to the initial condition and then senescence.

An ever increasing number of factors have been shown to be cyclical. The agglutination cycle is but one of many such cyclical changes in ageing germ cells. Its significance lies in part in the fact that quantitative values are more readily obtained in this than in many other characteristics.

Beginning with maturation there occurs a progressive improvement or increase until a maximum is attained. After this there is a progressive senescence or decrease in agglutinin production by the eggs and in agglutinability of the sperm.

This increasing phase in the life of the germ cells is too much ignored or not appreciated. The maximum reactivity of the egg, in respect to agglutinin production, fertilizability, segmentation, parthenogenesis occurs not when first matured but when partially overripe. This optimal stage may be synchronous with shedding or may occur many hours earlier or later. The determining factor is not the time after shedding, but the time after maturation, the OH ion concentration of the sea water and the temperature. If eggs are retained within the body for long periods, then only the decreasing phase is manifested.

The sperm undergoes a similar cyclical change. It has generally been assumed that agglutination time is determined by the amount of agglutinin, that the sperm is a constant, reacting in accordance with the dose of agglutinin.[1]

[1] Lillie, '14, does record that an increase of sperm was required to produce agglutination when the sperm were aged.

I have shown that "normal" sperm from different individuals are not in a similar condition even when freshly removed from freshly collected males at the height of the breeding season. They undergo, within the body of the male, all degrees of overripeness. Depending on the degree of overripeness will depend the duration of the agglutination reaction, when the other conditions are comparable.

When the sperm are recently matured, and tested at successive intervals, there is clear evidence of a progressive, marked yet slow increase in agglutinability followed by a rapid decrease. These changes in agglutinability occur even when the same egg waters are used, without eggs and without visible jelly. They may be due either to a substance which activates the egg to greater agglutinin production, or which activates agglutinin to greater activity, or a substance which makes the sperm more sensitive to a given dose of agglutinin.

That the substance is not secreted entirely by the eggs is shown by the large increase in agglutination when the same egg water was used at successive ages, and in which there were no eggs nor jelly. The possibility that dissolved jelly, containing agglutinin, may be in the egg water from old eggs, is practically eliminated. For at each age the eggs were carefully washed, and the subsequent procedure was the same.

It is improbable that agglutinin is activated or increased by the hypothetic substance. For aged sperm, which are no longer agglutinated by ripe eggs (with plenty of agglutinin), can be made to agglutinate by overripe eggs with little agglutinin.

The facts seem to point to a substance secreted by ageing sperm or a physiologic change in ageing sperm that makes them more agglutinable to a given dose of agglutinin.

The further analysis of the cause or causes of increased agglutinability is deferred to the next study.

One can hardly escape the conclusion that agglutination is not a function of a constant sperm reacting with the same intensity to a given quantity of agglutinin, but that agglutination is a function of many variables, including (1) cyclical increase, then decrease in agglutinin production by ageing eggs, (2) cyclical increase, then decrease in agglutinability of ageing sperm, (3) different rate of change in overripening eggs and of sperm.

Conclusions.

Ageing Eggs.

1. When freshly shed eggs were allowed to overripen, there was a progressive and a very marked increase in agglutination time. The maximum was reached in 3 to 5 hours. With further overripening there was a progressive decrease until the sperm were no longer agglutinated.

2. Eggs overripe when shed, and allowed to further overripen, disclosed little or none of the increasing phase. Only the second or decreasing phase was evidenced.

3. Overripeness within the body gave rise to the same kind and degree of change in eggs as overripening outside of the body.

4. The cyclical change in agglutination is due to an increasing liberation of agglutinin. With further overripening the decrease in agglutination is due not to disintegration and liberation of anti-agglutinins but to decreasing liberation of agglutinins. With yet further overripening the anti-agglutinins come into play.

5. The behavior of eggs from different individuals can be understood only when the exact physiologic condition of the germ cells is known, when shed.

6. The cyclical behavior of eggs in respect to agglutination is paralleled by other cyclical manifestations of overripening, such as rate of fertilization, membrane formation, rate of cleavage, etc.

Ageing Sperm.

1. Agglutination is conditioned not only by the degree of overripeness of the eggs but also of the sperm.

2. Sperm from freshly collected, freshly opened *Arbacia*, freshly prepared under strictly comparable conditions, and tested at each successive age by freshly shed eggs, agglutinated for increasing periods of time. The increase in agglutination began about the third hour, reached a maximum far beyond the initial values about the 24th hour, and thereafter decreased progressively.

3. When the sperm were overripe at the time of shedding, or precociously aged by high temperature, corresponding portions of the first or increasing phase did not take place. When more

overripe at the time of shedding, only the decreasing phase occurred.

4. The degree of overripeness of sperm at the time of shedding, as well as of eggs, was determined by a series of independent tests.

5. When conditions are strictly comparable the agglutinability of sperm depended upon the degree of overripeness of the sperm when shed, and with the degree of further overripening after shedding.

Ageing Eggs Tested by Ageing Sperm.

1. When the same eggs and sperm were used at successive ages, under strictly comparable conditions, not only was there a progressive and marked increase in agglutination values, but the increase was greater than with either overripe eggs or overripe sperm alone. This occurred provided the germ cells were freshly matured at the time of shedding.

2. When the sperm alone were overripe at the time of shedding there was an earlier increase.

3. When the eggs only were overripe at the time of shedding there was little or no change in values, due to insufficient agglutinin.

4. When eggs and sperm were both overripe at the time of shedding there was only the decreasing phase, due to insufficient agglutinin and to inability of sperm to change.

5. The agglutination phenomenon is conditioned by (*a*) the degree of overripeness of eggs and of sperm at the time of shedding, (*b*) the differences in the cyclical rate of change of sperm and of eggs, (*c*) time, (*d*) conditions of ageing, such as temperature, OH ion concentration.

6. Eggs increase in agglutinin production. Sperm increase in agglutinability with ageing. The two cycles are not synchronous. The resulting agglutination depends upon the extent of change in the eggs and in the sperm. The amount of change is a summation of these factors.

7. Sperm is not a fixed or neutral agent reacting in the same way to the same dose of agglutinin. Sperm is a varying agent, either secreting increasing amounts of a substance or substances

which accelerate agglutinin formation, or sperm undergoes a physiologic change, with overripening, as a result of which they become increasingly reactive to a given dose of agglutinin.

The analysis of this substance or physiologic change will be made in the next study.

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FACTORS THAT CHANGE AGGLUTINABILITY OF AGEING SPERM.

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By a previously described technique (Goldforb, '29*a*), agglutination of sperm by egg water could be measured with an average experimental error of 1 second or 4.5 per cent. With this technique freshly shed eggs from freshly collected and freshly tested sea urchins (*Arbacia punctulata*) were separately tested by freshly shed sperm, under strictly comparable conditions. These "normal" germ cells varied from 11 to 2,300 per cent. in agglutination time. This large variation was due in small part to germinal differences and in large part to wide differences in the degree of overripening of the germ cells at the time of shedding.

Later studies (Goldforb, '29*b*) showed that when eggs or sperm or both were not too overripe, at the time of shedding, there was, with ageing, a progressive and marked increase in agglutination time. The evidence compelled the conclusion that ageing eggs liberated increasing amounts of agglutinin, and that ageing sperm either secreted increasing amounts of a substance that increased the agglutination, or, that ageing sperm underwent a physiologic change that made them increasingly susceptible to a given dose of agglutinin.

The present study aims to determine which of these two possibilities actually obtains. The experiments were performed at the Marine Biological Laboratory at Woods Hole, Massachusetts, during the summers of 1924 and 1926. My thanks are due to the Directors for the facilities of the laboratory.

EXPERIMENTS WITH AGEING SPERM SUSPENSIONS.

In preliminary experiments, samples of the same sperm suspension and the same egg water solution were tested 10 to 50 minutes after the initial test. In a considerable number of instances the later test gave increased agglutination values. Exps. 5, 12, and 25 may serve as illustrations.

TABLE I.

SHOWS AN INCREASE IN AGGLUTINATION TIME WHEN SAMPLES OF THE SAME SPERM AND EGG CULTURES WERE USED 10 AND 20 MINUTES AFTER THE INITIAL TESTS.

Exp. No.	Age of			♀ No.	Agglutination in 1/80 Egg Water.				
	Eggs.	Sperm.	Egg Water.		Initial Test in Sec.	10 Min. Later		20 Min. Later	
						Sec.	% Increase.	Sec.	% Increase.
5*A*	4 hrs.	4 hrs.	1 hr.	1	11	13	18	19	72
				2	13	14	7	14	7
				3	7	7	0	7	0
				4, 5, 6, 7 [1]	12	16	33	21	75
B	1	22	21	1	24	26+	8		
				2	13	23+	76		
				3	8	26+	225	80	900
				4, 5, 6, 7 [1]	54	57	55	60	111
C	6½	22	11½	1	0	0	0		
				2	0	12	1200		
				3	0	13	1300		
				4, 5, 6, 7 [1]	17	17	0		
D_1	22	22	1	1	19	19	0		
				2	9	7	− 22		
				3	9	12	33		
				4, 5, 6, 7 [1]	12	0	0		
D_2	22	22 + 1 [2]	1	1	37	37	0		
				2	0	12	1200		
				3	12	13	7		
				4, 5, 6, 7 [1]	10	19	90		
D_3	22	1	1						
				2	13	12	− 7		
				3	17	11	− 35		
				4, 5, 6, 7 [1]	0	0	0		

[1] Eggs of females 4, 5, 6 and 7 were combined.
[2] Old and fresh sperm combined.

TABLE II.

SHOWS INCREASED AGGLUTINATION VALUES WHEN SAMPLES OF THE SAME OVERRIPE SPERM SUSPENSIONS ARE USED AT SUCCESSIVE INTERVALS.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.1	1 hr.	25 hrs.	25 hrs.	1	0				17	1700
				2	0				13	1300
				3	0				18	1800
				4, 5, 6	0				8	800
12.3	1	25	1	1	3			11		269
				2	0			5		500
				3	0			0		0
				4, 5, 6	0			10		1000
12.2	25	25	1	1	0			9		900
				2	0			12		1200
				3	0			0		0
				4, 5, 6	0			16		1600
12.4	1	1	25	1	22			0		− 2200
				2	42			17		− 60
				3	13			9		− 30
				4, 5, 6	17			20		17
12.5	25	1	1	1	22			15		25
				2	18			22+		22
				4, 5, 6	7			24		240

TABLE II.—(*Continued*).

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water Solution.					
	Eggs.	Sperm.	Egg Water.		Initial Test.	10 Min. Later.	15 Min. Later.	35 Min. Later.	55 Min. Later.	% Increase.
12.6	1	1	1	1	0			9		900
				2	6			0		− 600
				3	0			0		0
				4, 5, 6	17			11		− 35
25.3	0	6	1	1	0	0				0
				2	20	30				50
				3	18	23				27
				4	0	0				0
25.6	0	24	1	1	0		0			0
				2	0		28			2800
				3	0		16			1600
25.6	24	24	1	1	0		30			3000
				2	28		34			21
				3	47		40+			
25.6	0	3	1	1	0		11			1100
				2	24		28			16
				3	25		48			92
				4	12		43			258

In Experiment 5*A* (Table I) a fresh suspension of a 4 hour dry sperm was tested separately, with the egg waters of 4 females. The agglutination values were 11, 13, 7, and 12 seconds respectively. Ten minutes later samples of the same cultures gave 2, 1, 0, and 4 seconds *longer agglutinations* than the first tests, or an increase of only 16 per cent. Other samples of the same sperm suspension tested after 10 more minutes gave 8, 1, 0, and 9 seconds more than the initial tests, or an average *increase of 42 per cent.*

When a 22 hour sperm was used with freshly shed eggs (Exp. 5*B*) the initial agglutinations for the four females were 24, 13, 8, and 54 seconds respectively. Ten minutes later, samples of the same cultures gave increases of 2, 10, 18, and 3 seconds respectively, or 33 per cent. After 10 more minutes the increases were far greater, namely 126 per cent. When intermediate aged (6½ hours old) eggs were tested by 22 hours old sperm the first test gave 0, 0, 0, and 17 seconds, the second test 10 minutes later gave 0, 12, 13, and 17 seconds, *i.e.*, an increase of 147 per cent. When, however, freshly shed sperm was used (Exp. *D*3) no increase in agglutination occurred at the later test. When old and fresh sperm were combined (Exp. *D*2) there was again an increase in agglutination values, ten minutes later of 37 per cent.

Similar results occurred in Experiment 12 (Table II). Suspensions of a 25 hour dry sperm did not agglutinate with freshly shed eggs, but 55 minutes later agglutinated 17, 13, 18, and 8 seconds respectively (Exp. 12.1). In Experiment 12.3 the initial values were 3, 0, 0, and 0 seconds. After 35 minutes the values were 11, 5, 0, and 10 seconds respectively, an increase of 766 per cent. When 25 hour old eggs were used, the initial values were 0, 0, 0, and 0 seconds. The later values were 9, 12, 0, and 16 seconds (Exp. 12.2). Ten out of the twelve tests with the 25 hour old sperm gave material increases in agglutination.

On the other hand the freshly prepared suspensions of ripe sperm with ripe eggs, Exps. 12.4 and 12.6, gave little increased or much decreased agglutination. The average values were −13 per cent. and −61 per cent.

It appears that sperm in standard 1 per cent. suspension changed

within 10 minutes, changed further within the next 10 to 45 minutes, with corresponding increase in agglutination values. The change occurred much more markedly in overripe than in ripe sperm. The change occurred whenever the eggs were not so overripe that not enough agglutinin was liberated to activate and agglutinate the sperm.

In Exp. 25 similar results were obtained. The eggs and sperm were in good physiologic condition when shed. The sperm was used when 3, 6, and 24 hours old. Marked increases in agglutination occurred in 10 to 15 minutes after the sperm suspension was prepared. These increases occurred in 6 out of 9 tests. The other 3 tests gave no agglutination in either the first or the second tests. In other instances, when no agglutination occurred in the initial test, the second test gave long agglutinations. The average increases were 39, 39, 113, and 1466 per cent. respectively.

Other experiments corroborated these results and led to a more detailed study of ageing suspensions of sperm.

In Experiment 11*A* (Table III) both kinds of germ cells were six hours old. Six females were used. Females Nos. 1, 2, and 3 were tested separately, 4, 5, and 6 together. The temperature was 21° C. with an increase of ½° C. during the 2½ hours of the experiment. Tests were made 15 to 30 minutes apart, with samples of the same sperm suspension and of the same egg water solution. The successive agglutination tests for female No. 1 were 13, 15, 19, 19, 26, 19, 20, 20, 15, and 16 seconds respectively. *There was an unmistakable increase in values with ageing of the sperm suspension.* Maximum values with egg water of ♀1, occurred not when first tested but 75 minutes later, and the increase was 100 per cent. During the subsequent 75 minutes there was a slow and progressive decrease, which did not reach the initial values at the close of the experiment, 165 minutes later.

The egg waters of females No. 2, No. 3, and Nos. 4, 5, 6 combined gave similar results. The average values for the 4 batches of eggs were 14.5, 17.0, 19.2, 20.2, 19.5, 19.7, 17.0, 15.7, 14.0, and 11.5 seconds at the successive intervals. The increases were 100 per cent. for female 1, 56 per cent. for female 2, 35 per cent.

TABLE III.

INCREASE IN AGGLUTINATION WHEN SAMPLES OF THE SAME EGG WATER WERE TESTED BY SAME SPERM SUSPENSION AT SUCCESSIVE INTERVALS. TEMPERATURE 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water.										Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	15	30	50	75	90	105	120	150	165 Min.	
11*A*	6 hr.	6 hr.	1 hr.	1	13	15	19	19	*26*	19	20	20	15	16	100%
				2	16	19	22	21	18	*25*	22	20	19	18	56
				3	14	14	15	15	*18*	6	7	9	7	0	35
				4, 5, 6	15	20	21	26	21	*29*	19	14	15	12	93
				Aver. sec.	14.5	17.0	19.2	20.2	19.5	19.7	17.0	15.7	14.0	11.5	71
				Aver. inc. %		+ 17	+ 31	+ 39	+ 34	+ 36	+ 17	+ 8	− 3	−20	
11*B*	6	31	6	1	0	0	0								
				2	0	0	0								
				3	10	0	0								
				4, 5, 6	16	0	0								

					Agglutination in Sec. in 1/80 Egg Water. Temp. 20½° C.								Maximal Increase.
					0	5	15	25	50	80	100	125 Min.	
8*A*	5	29	1	1	21	50	40	70	*90* +	0			328%
				2	17	0	0	0	0	0			
				3	34	40	50 +	90 +	*95* +	6			179
				Aver. 1 & 3	27.5	35	45	80	92	0			
				Aver. inc. %		+ 27	+ 63	+ 191	+ 236	− 89			
				Sea-water					0	0			
8*B*	5	6	1[1]	1	24		24	29	34	39	37	*63*	120
				2	14		*17*	8 +	11	9	11		
				3	23		22	30	29	29	52	76	230
				Aver. 1 & 3	23.5		23	29.5	31.5	34	44.5	64.5	
				Aver. inc. %			− 2	+ 25	+ 34	+ 44	+ 89	+ 174	

[1] Not same egg water solution as in 8*A*.

TABLE III.—(*Continued*).

RANGE OF TEMPERATURE FROM 20½° C. TO 20° C.

Exp. No.	Age of			♀ No.	Agglutination in Sec. in 1/320 Egg Water at																	Maximal Increase.
	Eggs.	Sperm.	Egg Water.		0	10	20	30	45	55	70	100	120	140	160	180	200	240	260	280	305 Min.	
14*A*	1 hr.	24 hr.	1 hr.	1	30	30	36	48	53	45	60	70	80	62	42	36	26	3	0	0	0	100%
				2	26	26	28	31	43	40	46	42	40	30	27	24	19	6	0	0	0	77
				Aver.	23	23	32	39.5	48	42.5	53	56	60	46	34.5	30	22.5	4.5	0	0	0	
				Aver. inc. %	0	0	+39	+72	+108	+85	+130	+143	+160	+56	+50	+32	−2	−80	−100	−100	−100	
B[1]	1	1	1	1	29	28	21	19	20		18	17	15	11	12	11	12	10	10	10	10	0
				2	21	25	26	23	23		20	21	20	16	15	16	16	16	15	11	11	24
				Aver.	25	26.5	23.5	21	21.5		19.5	19 5	17.5	13.5	13.5	13.5	14	13	12.5	10.5	10.5	
				Aver. change %	0	+6	−6	−16	−14		−22	−22	−30	−46	−46	−46	−44	−48	−50	−58	−58	

[1] Same egg water as in *A*.

for female 3, and 93 per cent. for females 4, 5, and 6. The average increase was 71 per cent. In every instance the maximal values did not take place at the initial test but 75 to 90 minutes later. When the experiment was terminated, after 165 minutes, the values were greater than the initial test in 2 batches,

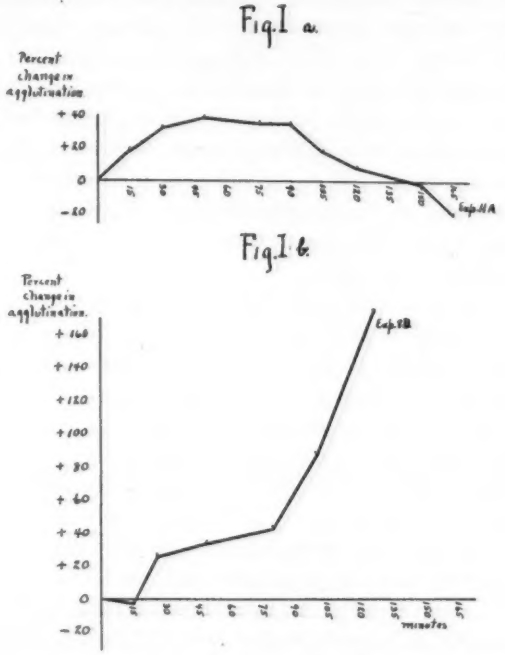

FIG. 1. Shows the slow progressive *decrease* in agglutination values when a suspension of freshly shed sperm is tested at successive intervals with samples of the same egg water.

slightly lower in 1 batch, and in 1 batch agglutination had ceased altogether (Fig. 1*a*).

In Experiment 8*B* the eggs were 5 hours old. These were tested by 2 kinds of dry sperm, one 6 hours old and the other 29 hours old. The temperature increased but $\frac{1}{2}$° C. during the 2 hours of the experiment. When a suspension[1] of the 6 hour dry sperm was tested with the egg water of female No. 1, the

[1] All suspensions of sperm were 1 per cent. and tested immediately.

successive values were 24, 24, 29, 34, 39, 37, and 53+ seconds, respectively. A similar increase occurred with the egg water of female No. 3, namely 23, 22, 30, 29, 29, 52, and 76 seconds. The eggs of female No. 2 were very overripe at the time of shedding, as indicated by enlarged size, oval shape, pale color, greater viscosity, rate of membrane formation, etc. The egg water of these overripe eggs gave a very small increase, then decreased in value, namely 14, 17, 8+, 11, 9, and 11 seconds.

When eggs were not too overripe at the time of shedding, as in female No. 1 and No. 3, there was a progressive and marked increase in values with ageing of sperm suspension, namely 120 per cent. for female No. 1, 230 per cent. in female No. 3. The increase began 10 to 25 minutes after the initial test. The maximum values were not reached during the 125 minutes of the experiment (Fig. 1*b*).

Other samples of the same egg water were tested by a sperm suspension made with a 29 hour dry sperm (Exp. 8*A*). The agglutination values increased to a far greater extent than with the less overripe sperm of the previous experiment. This is in entire accord with the results obtained in ageing dry sperm (Goldforb, '29*b*). The values with the egg water of female No. 1 were 21, 30+, 40+, 70+, 90, and 0 seconds respectively. Female No. 3 gave 34, 40, 50+, 90+, 95+, and 6 seconds respectively. The increases were 328 and 179 per cent. Maximum values occurred in both, 50 minutes after the initial test. There was a very rapid decrease in values after this maximum. Female No. 2, with the very overripe eggs, gave a 17 second agglutination in the first test but no agglutination thereafter (Fig. 2).

It may be concluded from these experiments that *when eggs and sperm were not too overripe, when shed, agglutination progressively increased with ageing of the sperm suspension. The increase was more rapid and reached a greater maximum, the more overripe the dry sperm at the initial test. The change in values cannot be attributed to a change in concentration of sperm suspension, nor to a difference in agglutinin content, nor to ageing of egg water solution.* For, when such egg water was tested at successive intervals by freshly shed, freshly prepared sperm, there was no progressive increase in agglutination values. Maximal

agglutination with ageing sperm occurred not at the initial test but 50 to 125 minutes later. The increase was 35 to 328 per cent.

Ageing sperm suspensions gave a similar, progressive and marked increase in agglutination values as did ageing of dry sperm (Goldforb, '29*b*). Ageing sperm suspensions gave, however, a much quicker increase.

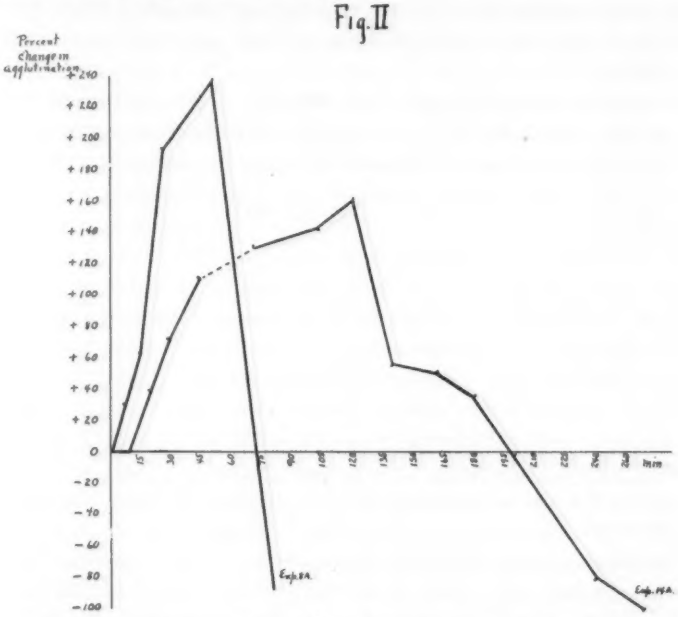

FIG. 2. Shows the slowly *increasing* agglutination values, when partially overripe (6 hours old) dry sperm is used.

In Experiment 14*A*, the ageing sperm suspension was tested for a longer period (305 minutes). Two kinds of sperm were used, freshly shed and 24 hour dry sperm. These were tested by the same egg water solutions from the freshly shed eggs of two females. The egg water was more diluted (1/320) than in the other experiments, which made for greater accuracy. The temperature changed but ½° C. during the five hours of the experiment. Successive tests were made 10 to 25 minutes apart.

The results obtained with the different samples of the same

ageing suspensions of overripe (24 hour) dry sperm conform in all essentials with those in previous experiments. There was a marked and progressive increase in agglutination with ageing of sperm suspension. This increase began 10 to 20 minutes after the initial tests. Maximal values were reached 120 and 70 minutes after the first tests, and were 166 and 77 per cent. greater. Thereafter the values decreased steadily. When the sperm suspension was 260 minutes old, agglutination ceased (Fig. 2).

The parallel experiment with samples of the same egg water solution but tested with non-overripe (freshly shed) sperm gave very different results. Female No. 1 gave at successive intervals

FIG. 3. These curves represent the behavior of dry sperm 24 and 29 hours old. The ageing suspension gave rise to a *more rapid and greater increase* than 6 hour dry sperm. The more rapid and greater increase in 8*A* may be due in part to the older dry sperm.

no progressive increase as in the case of overripe sperm, but on the contrary a progressive decrease, as described by Lillie, '14, '15, Cohn, '18, Lillie and Just, '24. The values were 29, 28, 21, 19, 20, 18, 17, 15, 11, 12, 11, 12, 10, 10, 10, and 10 seconds respectively. The values for female No. 2 showed the same progressive decrease, after a brief, small increase. This small and early increase of 6 per cent. is probably of no significance. The values were 21, 25, 26, 23, 23, 20, 21, 20, 16, 15, 16, 16, 16, 15, 11, and 11 seconds respectively (Fig. 3).

Sperm too overripe, *i.e.*, 31 hour dry sperm (Exp. 11) did not agglutinate at all.

The data are plotted in Figs. 1 to 3.

The increase in agglutination either did not occur or only slightly, when the dry sperm were not overripe at the beginning of the experiment. Nor did it occur when the sperm or the

eggs were so senescent that agglutination did not occur at all, as in Exp. 11*B* and in female No. 2 of Exp. 8*A*. But agglutination did increase when the dry sperm was in intermediate stages of overripeness.

The close agreement in the results with ageing dry sperm (Goldforb, '29*b*) and with ageing sperm suspensions is most striking. The difference lies only in the rate of increase which is so much faster in ageing sperm suspensions than in ageing dry sperm.[1]

It is known that sea water dilutes the H ion concentration of the sperm culture, thereby activating the sperm. But activation by sea water neither gave rise to, nor increased, the agglutination values. Hence the increase in values with ageing of sperm suspension must be attributed to causes other than H ion concentration *per se*.

In searching for the cause or causes of this phenomenon I have excluded an increase in agglutinin as a factor. For the same egg water was used in successive tests, and there were neither eggs nor visible jelly in such solutions. I have excluded the effect ageing of the egg water solution. For when such egg water solution was tested at each successive interval by freshly prepared ripe sperm there was no progressive increase in agglutination values. Temperature was eliminated as a factor, for not only was the change but ½° C., but the increase in agglutination occurred both when the temperature increased and when it decreased. A change in concentration of sea water was also eliminated.

The factor or factors that made for increasing agglutination must be sought in the sperm. If the sperm secretes a substance with ageing, it is not one which activates the eggs to greater agglutination liberation. For there were no eggs in the solution. Nor is there any evidence that it progressively activates the agglutinin in the solution. The other possibility is that sperm undergoes with ageing a physiologic alteration which makes the sperm increasingly susceptible to a given dose of agglutinin. The change is a cyclical one increasing with age, reaching a maximum long after the initial test, and decreasing with further ageing.

[1] Drzewina, A., and Bohn, G. ('26), found that an increasing number of eggs (*Strongylocentrotus*) develop when dilute sperm stood for a time.

The parallelism between the known physiologic changes and ageing of sperm is most striking, such as longevity, viscosity, mobility, metabolism, permeability, agglutination. These physiologic changes are all cyclical. The agglutination increases with overripening of sperm either dry or in suspension. The dry sperm undergoes slower physiologic changes than do sperm in suspension, and shows correspondingly slower increase in agglutination values.

Is There an Increase of Sperm Substance in Increasing Concentrations of Sperm?

A number of investigators have successfully extracted a substance or group of substances from sperm. Winkler, '00, Robertson, '12, Foa, '18, Prevost and Dumas, '24, extracted a sperm substance which induced parthenogenesis. Sampson, '26, described the chemico-physical properties of a sperm filtrate which induced parthenogenesis. Dubois, '00, extracted a spermase, Ostwald, '07, a peroxidase and a catalaze. Geis, '01, and Loeb, '06, were unable to find enzyme characteristics in the sperm extract, but Richards and Woodward, '16, did. Lillie, '15, and Cohn, '18, suggested the possibility that overripe sperm may liberate a substance which aids in fertilization. Popa, '27, described a lipochromatic substance in the sperm head, which substance he believes responsible for agglutination.

Such investigations strongly suggested that overripe sperm may secrete a substance which modifies agglutination. While the evidence from ageing sperm strongly pointed to a physiologic change in the sperm, the hypothesis of increasing liberation of sperm substances with overripening was not excluded.

To find out whether the sperm liberated a substance which progressively increased agglutination, experiments were made with increasing concentrations of sperm, but with samples of the same egg water. The concentrations of sperm ranged from $\frac{1}{2}$ per cent. to 25 per cent. More than 25 per cent. could not be used for the suspensions were then too opaque to distinguish agglutinated clusters in the thick creamy mass of sperm. If the increase in agglutination in previous experiments was due to an increasing liberation of a sperm substance which energized

agglutinin, one should expect that increasing concentrations of sperm should correspondingly lengthen agglutination time. If the increase is due to a physiologic change in the sperm, then an increase in concentration, *per se*, should not increase agglutination.

In Experiment 4*A* (Table IV) ripe sperm was used in ½, 1, 2, and 4 per cent. suspensions. The agglutination values in a 1/20 dilution of egg water were 19, 27, 25, 28 seconds respectively. In a 1/60 egg water dilution, and therefore more accurate, with sperm in ½, 1, 2, 4, 10, and 25 per cent. concentrations, the agglutination values were 7, 14, 13, 13, 13, and 13 seconds respectively. In a still more dilute egg water dilution, namely 1/120, and with sperm in 1, 2, 4, and 10 per cent. concentrations, the values were 9, 9, 10, and 9 seconds respectively. The egg water of female No. 2 in a dilution of 1/120 gave 17, 27, 22+, 27, 28, and 28 seconds.

Less than 1 per cent. concentration of sperm did not give full agglutination values as Lillie has already made clear. At least 1 per cent. suspension is needed. But increasing the concentration of sperm *did not* further increase the agglutination values even in the dense 25 per cent. concentration.

There was the possibility that not enough time had been allowed for the liberation of the hypothetical substance or substances from the sperm. Previous experiments, however, showed that ageing 1 per cent. sperm suspension began to increase in agglutinability within 5 to 25 minutes, and reached maximal values in 15 to 125 minutes. Ageing dry sperm showed the initial increase within 3 hours and gave maximal values in 63 per cent. of the tests when 3 to 4 hours old.

In Experiment 4*B*, therefore, the same dry sperm was used as in the previous experiment, but was now 3½ hours old. Furthermore the sperm suspension was 65 minutes old when used. This was then deemed ample time to liberate the hypothetic sperm substance. With increasing concentration of sperm there should be increasing quantities of the sperm substance, manifested in increasing agglutination values. In a 1/20 dilution of egg water and with 1, 2, 4 per cent. sperm concentrations the values were 28, 27, and 29 seconds respectively. In the more delicate tests

TABLE IV.

SHOWING NO INCREASE IN AGGLUTINATION WITH INCREASING CONCENTRATION OF SPERM. AGGLUTINATION IN SECONDS.

Exp. No.	Age of			♀ No.	Concentration of Sperm in %.						Egg Water Dilution.
	Eggs.	Sperm.	Egg Water.		½	1	2	4	10	25	
4A	1½ hr.	1½ hr.	1 hr.	1	19	27	25	28			1/20
				1	7	14	13	13	13	13	1/60
				1		9	9	10	9		1/120
				2	17	27	22+	27	28	28	1/120
4B	3½	3½	1	1		28	27	29			1/20 Suspension 1 hr. old.
				1		10	9	11	11		1/120 " " " "
19A	1	1[1]	1	1a	28	26	27	26	27	28	1/320 Aliquot parts
				1b	27	29	29	28	27	28	1/320 of eggs.
19B	1	1[1]	26	1	17	19	18	18	19	18	1/320
				2	11	20	19	20	21	21	1/320
19C	26	1[1]	1	1	17	18	18	17	18	20	1/320
				2	19	20	15+	16+	16+	18	1/320

[1] Sperm overripe when shed.

with a 1/120 egg water dilution, and with sperm concentrations of 1, 2, 4, and 10 per cent., the values were 10, 9, 11, and 11 seconds. There was clearly no evidence of a sperm substance, even after the lapse of so much time, in any of the concentrations of sperm used.

Experiment 19 is an example of another type of experiment. The sperm, when shed, were by various tests shown to be overripe (Goldforb, '29*a*, '29*b*). This overripe sperm was then tested by (*a*) egg water from freshly shed ripe eggs, (*b*) other freshly shed ripe eggs whose egg water was 26 hours old, (*c*) fresh egg water from overripe (26 hour old) eggs. If a substance is liberated by overripe sperm, it should manifest its presence in one or more of these three tests with overripe sperm. The egg water dilution was 1/320 throughout. The temperature was 22½° C. The sperm concentrations were ½, 1, 2, 4, 10, and 25 per cent., freshly prepared for each test.

In Experiment 19*A* the freshly shed ripe eggs were divided into two equal portions. The egg water of each was tested separately with freshly prepared sperm suspensions. This served to check the accuracy of the experimental method. One portion gave, in increasing concentrations of sperm, 28, 26, 27, 26, 27, and 28 seconds, the other registered 27, 29, 29, 28, 27, and 28 seconds. There is a remarkably close agreement in the two samples of eggs. There was clearly no evidence of a sperm substance which increased the duration of the agglutination phenomenon.

Comparison of Experiments 19*A*, 4*A*, and 4*B* with those experiments in which ageing sperm were used, brings out in sharpest relief the complete absence of any increase in agglutination in either ripe or in overripe sperm by merely increasing the concentration of the sperm, while progressive and marked increases occurred in 1 per cent. suspensions as they became increasingly overripe.

In Experiment 19*B* old egg water (26 hours old) was used, with the same overripe sperm, in the same concentrations. The egg water of female No. 1 gave 17, 19, 18, 18, 19, and 18 seconds respectively. Female No. 2 gave an 11 second reaction in ½ per cent. sperm suspension, and 20, 19, 20, 21 and 21, seconds in

the other concentrations. The heavy concentrations of sperm cultures did not manifest any increase in agglutination in these old egg water solutions.

In Experiment 19*C* overripe (26 hour) eggs were used. Female No. 1 gave, in the increasing sperm concentrations, 17, 18, 18, 17, 18, and 20 seconds. Female No. 2 gave 19, 20, 15+, 16+, 16+, and 18 seconds. There was no increase in values when overripe eggs were used.

In Experiment 19 with ripe or overripe eggs, with fresh and with old egg water solutions, with ripe or overripe sperm, *there is no evidence of a substance liberated by sperm.*

Discussion.

There can be no doubt that agglutination was not increased with increasing concentration of sperm. On the other hand agglutination was markedly and progressively increased by ageing of sperm, either concentrated or in suspension. When increased agglutination took place, it was *not* due to a substance activating the eggs to greater agglutinin production. For the increase in agglutination values occurred in samples of the same egg water from which eggs and jelly were excluded.

It is conceivable that the substance may be modified so as to intensify the activity of the agglutinin in the egg water. This was suggested as a possibility by F. Lillie, '19. In the first place there is no known basis for this hypothesis. Much more pertinent is the fact that one should expect on this hypothesis an increase in agglutination values in those experiments in which increasing concentrations of sperm were used. But no such increase occurred.

It should be recalled that students of the agglutination phenomenon in bacteria have come to a similar conclusion, namely, that a physiologic change or changes in the bacteria are responsible for the change in agglutination (McGregor, '10, Ficai, '12, Kabeshima, '13, Buchanan, '19).

The observation that loss of fertilization occurs more quickly than loss of motility (Lillie, F., '14, '15, '19, Lillie, F., and Just, E., '24) is paralleled by the observed fact that loss of agglutination occurs more quickly than loss of motility. It is conceivable

and probable that loss of fertilization and of agglutination are associated with the physiologic changes described above.

Sufficient time elapsed to permit the substance, if present, to be liberated into the culture medium. Ripe eggs liberate a substance, agglutinin, in about 15 minutes. Sperm on account of their small size and relatively large surface should liberate their substance more quickly. Yet in 3½ hours, ripe dry sperm did not give any evidence of a substance that increases agglutination, nor did the overripe sperm in Experiments 19*A*, 19*B*, 19*C*.

Nor may one assume that the hypothetic activating substance is formed and liberated increasingly with ageing sperm. For even in overripe sperm, Experiment 4*B*, the increasing concentrations give no evidence of increase in agglutination.

It is known that sperm give off CO_2, and the carbonic acid thus formed modifies the relative OH ion concentration of the medium. The work of Loeb, '03, '14, Lillie, '14, '15, Cohn, 18, Lillie and Just, '24, clearly indicates that the carbonic acid' plays an important rôle in *decreasing the activity and thereby increasing the longevity of the sperm.* It is known that *carbonic acid induces aggregation but there is no evidence of carbonic acid increasing agglutination.*

It is therefore concluded that carbonic acid is not responsible for the increasing agglutination values.

It is furthermore concluded that there is no definite evidence of a substance liberated by sperm which increases the intensity or the duration of agglutination.

The facts strongly suggest a cyclical physiologic change or changes in the sperm, which make the sperm more reactive to a given dose of agglutinin.

It is known that both eggs and sperm undergo cyclical changes. This is evidenced by changes in metabolism, in gelation of surface, in viscosity, in permeability, etc., all of which are associated with overripening. Overripening eggs show progressive solution of jelly, liberation of agglutinin, or increased rate of agglutinin production. Hence overripening eggs give rise to increased agglutination values. Overripening sperm, undergoing similar physiologic cyclical changes, increases in agglutinability.

When therefore ageing eggs are tested by ageing sperm the agglutination values are greater than when either eggs or sperm alone are aged. This is true not only for ageing dry sperm but for ageing sperm suspensions.

It is this physiologic change in sperm as well as in eggs that gives rise to the initial improving stage, evidenced here in increasing agglutination. This physiologic change, continuing, leads to an optimum condition of the germ cells and then to senescence.

The cyclical physiologic change in eggs is manifested by the cyclical increase then decrease in agglutinin liberation. The cyclical physiologic change in sperm is manifested by increase then decrease in agglutinability.

The life cycle of the sperm may be abbreviated, *i.e.*, the physiologic changes may be hastened by dilution, by heat, by excess OH ions, etc. With such increase there is a corresponding precocious increase in agglutinability. Freshly matured dry sperm may not show evidences of a change for 3 hours. Overripe dry sperm show evidences of a physiologic change at once. Suspensions of ripe sperm show little evidence of a physiologic change during the whole life of the sperm.[1] Moderately overripe sperm in the same dilution show evidences of a physiologic change in 15 to 25 minutes (Exp. 8*B*, 11*A*). Sperm more overripe show evidence of a physiologic change sooner, namely, 5 to 20 minutes (Exp. 8*A*, 14*A*).

If the factor which gives rise to increased agglutinability be a sperm secretion, there should occur with increasing concentration of sperm correspondingly increased agglutination. This does not take place. On the other hand, if the factor be a physiologic change in the sperm, then increasing concentration of freshly prepared sperm should produce no change in agglutination, which is exactly what takes place.

I am therefore compelled to conclude that the increase in agglutination so marked in ageing sperm, whether dry or in suspension, is due to a physiologic change in the sperm, which change makes them more susceptible to a given dose of agglutinin.

[1] It is improbable that the change may occur within the first 10 minutes.

The discovery of agglutinins secreted by the eggs led to the assumption that the egg plays the dominant rôle in agglutination.[1] Lillie, '17, dealing with other phases of germ cell behavior, states that "the old idea that sperm supplies organs or substances necessary for activation must be abandoned. The egg possesses all substances needed for activation. The sperm is an inciting cause of these reactions within the egg system. . . ." It has been assumed that secretions of varying amounts of agglutinin were the determining factor in changing agglutination. The sperm merely reacted to varying quantities of agglutinin.

My studies have shown that the egg does not decrease, but on the contrary *increases the rate of agglutinin liberation* with age until an optimum is reached several hours after maturation. These studies also showed that *sperm are not constant* as heretofore assumed, but that sperm is equally variable, increasing in agglutinability with overripening, until an optimum is reached 6 to 24 hours after maturation (dry sperm) or 70 to 120 minutes after preparation of the suspension. This cyclical change in agglutinability appears not to be due to a secretion, but to a physiologic change which makes sperm increasingly agglutinable by a given dose of agglutinin.

Summary.

Previous studies demonstrated that with increasing overripening of eggs or of sperm, or both, agglutination values increased correspondingly.

The present study demonstrates that precocious overripening of sperm, by dilution, gave rise to a correspondingly precocious and markedly progressive increase in agglutination.

This precocious increase occurred when the dry sperm were overripe. The increase began in 5 to 20 minutes after the initial test. Maximum values occurred 15 to 125 minutes after the initial test. The increase in values ranged from 77 to 328 per cent. The greater the overripeness of the dry sperm the greater the increase, the earlier the maximum and the sooner the cycle ended.

[1] For full bibliography and review I refer to Lillie, Problems of Fertilization, '19, Lillie and Just in General Cytology, '24, and to Morgan, Experimental Embryology, '27.

Suspensions made of ripe sperm either did not increase at all or only slightly. The agglutination values then decreased progressively. This is the phase heretofore described.

The cyclical increase and decrease in agglutination was not due to a change in the eggs, nor jelly, nor temperature, nor to a changed OH ion concentration.

This increase in agglutination is not due to a substance liberated by sperm. For agglutination values were not increased when the concentration of sperm was increased from 1 to 25 per cent., the maximum concentration usable. The values were the same whether ripe or overripe sperm were used.

Sufficient time elapsed for the substance, if present, to be liberated.

The CO_2 liberated by sperm has considerable effect upon the activity of the sperm, upon aggregation, but does not increase agglutination.

The cyclical agglutination change is due to a physiologic change in ageing sperm, manifested in a changing metabolism, gelation, viscosity, permeability, and in an increased reaction of sperm to a given dose of agglutinin.

This cyclical change is paralleled by the eggs, which increases agglutinin liberation.

It is therefore concluded that the cyclical physiologic change in overripening is responsible for the improving phase in both germ cells, and with subsequent senescence. This physiologic change is analogous to that in agglutinating bacteria.

Sperm are not a biologic constant, as heretofore believed, but undergo marked physiologic changes with corresponding marked and progressive increase in agglutinability followed by progressively decreasing agglutinability.

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A NEW CASE OF INTERSEXUALITY IN *RANA CANTABRIGENSIS*.[1]

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The specimen to be described is an adult sexually mature wood-frog, *Rana cantabrigensis*, killed on October 26, 1928. It measures 3.7 cm. from the tip of the snout to the ischial symphysis, and is definitely and typically male in all the somatic features which are characteristic of this sex in this species. Nuptial pads are distinct and pigmented, very similar to those found in normal male frogs during this season, and the Wolffian ducts possess well-defined seminal vesicles in the usual position. The gonads appear as normal testes, which, upon a general macroscopical examination, show nothing atypical, nor any external evidence of germinal intersexuality.

Upon sectioning, both gonads are shown to be intersexual. The bulk of either gonad is composed of spermatic tissue, the histological structure of which corresponds exactly with that found in normal testes of this season. Most of the seminal tubules are thickly lined with dense bundles of spermatozoa. Examination of numerous male sex cells does not show any deviation from the normal processes of development.

The ovarian elements consist of individual ova or oöcytes, which may be designated as testis-ova (Fig. 1). The left gonad contains only one testis-ovum, deeply imbedded in the spermatic substance, while in the right gonad there is about a dozen of such cells scattered throughout. All of the ova are actually situated within the seminal tubules and lie among the male germ cells. None of them is associated with pigmentation, nor with any tissue hypertrophy, both of which are characteristic features of certain degenerative changes of germinal elements. The ova average about 200 micra in diameter, the larger ones measuring 250 micra or more. In shape the ova differ considerably, being mostly round or oval. In many places, they become much distorted in form, owing to the pressure exerted on them

[1] Contribution from the Zoölogical Laboratory of the University of Michigan.

by the tubules in which they are contained. Occasionally they occupy the whole lumen of the tubule, causing some derangement of the male germ cells nearby. Structurally, the testis-ova are devoid of true ovarian follicles: they appear invested by a

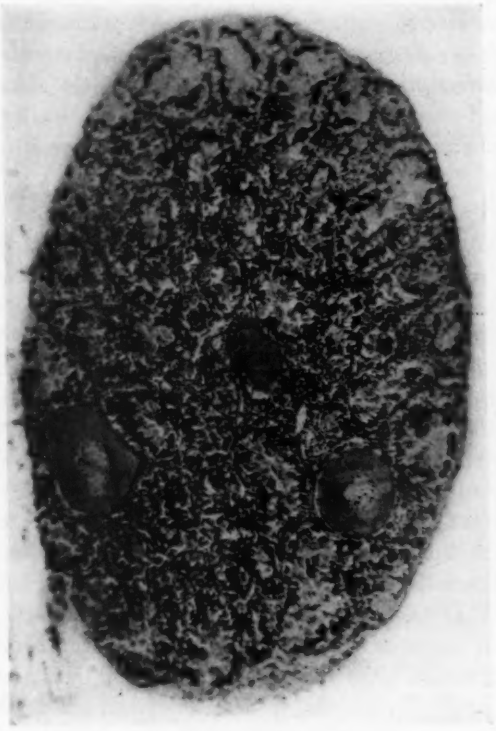

FIG. 1. A cross section of the right abnormal testis, showing three ova within the seminal tubules. Magnification approximately × 64.

delicate non-cellular fibrous membrane. Their nuclei are distinct, with sharply defined nuclear membranes, though thin and often irregular. There is no visible evidence of any intranuclear network. The nucleus stains only slightly, while the numerous nucleoli of various sizes are intensely basophilic, aggregating mostly along the periphery. Minute granules are found imbedded everywhere in the nucleus. The oöplasm stains grayish

and shows no vitellogenetic activity. All structures and conditions indicate a remarkable resemblance of these testis-ova to the normal young oöcytes of similar size.

From the above description, it appears that the testis-ova are of no functional value. Should they mature, there is no means of exit to the exterior, for there is no identifiable trace of Müllerian ducts. On the other hand, the presence of well-developed nuptial pads and of a perfect male accessory sex apparatus warrants the conclusion that this abnormal frog must have functioned as a male, despite the duplex condition of its gonads.

Review of Literature.

Instances of ova-containing testes have been encountered in various species of *Rana*.[1] Balbiani wrote (1879, p. 219), "Il arrive assez souvent, lorsqu'on pratique des corpes de testicules de Grenouille ou de Crapaud, même parvenus à l'âge de reproduction, de trouver dans les tubes ou les ampoules séminifères des ovules anormalement developpés, constitués identiquement comme les jeunes ovules transparents de l'ovaire de la femelle." Pflüger (1882, p. 33) found pronounced "Graaf'schen Follikel" in the testes of three-year-old Utrecht frogs. According to Spengel (1884, p. 270), Fritz Meyer had observed in the Leipzig frogs, "Hoden mit Ovarialeinschlüssen und umgekehrt Ovarien mit Hodeneinschlüssen." Marshall (1884) reported several abnormal frogs (*Rana temporaria*), one of which possessed ova-containing testes as well as Müllerian ducts. Another specimen of similar nature in the same species was found by Latter (1890). In *Rana viridis*, Friedmann (1898 *a*) described well-developed as well as degenerating ova in otherwise normal adult testes. He also observed (1898 *b*, p. 874) in the same species, "in mehreren Hoden intratubulär gelegene Gebilde, die zumindesten eine grosse Aehnlichkeit mit jungen Eizellen aufweisen." Mitrophanow (1894) noticed one true ovum and many doubtful ones in the testes of a frog (*Rana esculenta*). From references given by him and Cole (1896), Eismond (1892) had a young male frog showing testis-ova in various stages of development. In *Rana*

[1] Some of the cases cited below may be examples of Pflüger's hermaphroditism, which is found to be of normal occurrence in certain races of frogs.

pipiens, King (1910, p. 166) reported rudimentary ova in an adult testis and Swingle (1917) large oöcytes in well-developed testes of a young "pseudo-hermaphrodite." Champy (1913, p. 103) frequently observed oviform cells in testes of *Rana temporaria* and *Rana esculenta*. Witschi (1914, p. 69; 1921, pp. 326, 328) presented cases of testis-ovum in immature *Rana temporaria*. Takahashi (1919, p. 310) found, in an otherwise normal adult male of *Rana limnocharis*, three egg-cells in the right gonad. Van Oordt (1923) discovered about 100 ova of 100–160 micra in diameter in the right testis of an adult frog (*Rana fusca*). In *Rana catesbiana*, Swingle (1926, pp. 473–474, 491, 492; Fig. 97) mentioned the occurrence of maturation cells (which are probably ovarian) in larval testes, and of oöcyte-like cells in larval and adult testes. In *Rana sylvatica*, Witschi (1929, p. 267) found young oöcytes in the early development of male gonals. Cheng (1930) described a number of intersexual gonads in tadpoles of *Rana cantabrigensis*, which exhibited varying amounts of female substance amid male tissues.

Marshall (1884), Heymons (1917), Crew (1921 *a*), and Lloyd (1929) reported cases, in which one or both of the testes, otherwise normal, were found to contain pigmented materials. These pigmented bodies are often interpreted as degenerating ova or remains of ovarian tissue. Chidester (1926) recorded two interesting specimens of *Rana catesbiana*, in which ova were found in other parts of the body, instead of in the testes, which were normal in these cases.

There is always some difficulty in making a clear-cut distinction between an ovo-testis (or ovotestis) and an ovum- or ova-containing testis. The writer has regarded an ovotestis as composed of recognizable portions of ovary and testis, while an ovum- or ova-containing testis is mainly testicular in structure, containing an ovum, ova or groups of ova in its spermatic substance. Intermediates between the two types have been encountered and can be arranged in a genetical series. There are cases on record in which one gonad is an ova-containing testis, while the other one is a more or less distinct ovotestis (Kent, 1885;[1] Cole, 1896; Crew, 1921 *a;* Woronzowa, 1926, and others).[2]

[1] See Crew's re-description of Kent's specimen (Crew, 1921 *a*).

[2] Recently Christensen (1929) and Witschi (1929*b*) have reported new cases of intersexuality in *Rana pipiens* and in *Rana temporaria* respectively.

In toads, testis-ova have been recorded by Balbiano (1879), Hoffmann (1886), Knappe (1886), Cole (1896),[1] Ikeda (1896), Friedmann (1898 *a*), Cerruti (1907), Champy (1913), Takahashi (1915, 1919), Caroli (1925, 1926, 1927), Beccari (1925), Cunningham and Becton (1926), and Stohler (1928). In *Hyla*, Sweet (1908) found, in an adult male, imperfectly developed Müllerian ducts and 15–20 ova scattered irregularly through the substance of the testes, generally singly but sometimes in groups of two.

Discussion.

Popoff (1909) observes in the testicular tubules what he designates as "ovules mâles," each one of which eventually gives rise to "une spermatogemme et ensuite à un faisceau spermatique définitif." Swingle has argued that oöcyte-like cells are not necessarily female sex cells, especially when occurring in an otherwise male individual. Levy (1920) and Orlowski (1925) claim that the so-called eggs in the testicular tissue are nothing but "polyploide Riesenzellen." Champy (1913) presents some evidence to show that the primitive gonia in the male gonad may sometimes become hypertrophied to form oviform cells which may develop further and "aboutit à la formation d'ovocytes incontestables qu'on ne peut distinquer de ceux d'un jeune ovaire." Crew and Fell (1922) are of opinion that the ovum-like bodies in their material are mere liquefaction products of the spermatozoa.

In our specimen, the testis-ova obviously cannot be homologized with the "ovules mâles" of Popoff. They do not represent any stage in the normal development of male sex cells and there is no trace of anything resembling them in sections of normal testes of our wood-frogs. The size and morphology of the testis-ova show distinctly that they cannot be "polyploide Riesenzellen," nor coalesced masses of degenerating spermatozoa. Finally, there is no indication in the histology of the ovum-containing testes nor in the cytogenesis of the sex cells contained therein, that the testis-ova have been derived by an abnormal hypertrophy of the spermatic substance. No intermediate or

[1] Cole stated that Dr. Beard had informed him that well-developed ova occurred apparently normally in the testis of the toad quite apart from the Bidder's organ.

transitional forms exist between the testis-ova and the male sex elements. From these considerations we may reasonably assume that the testis-ova in our frog are true ovarian cells, though immature and apparently degenerating.

The occurrence of female germ cells in the testes described in this paper definitely demonstrates that male and female sex elements can, under certain circumstances, become developed and fostered in one and the same gonad of the frog. It affords additional evidence of a germinal bipotentiality, owing to which the indifferent germ cells, in the gonad of one sex, may change their course of development and assume the characteristics of the other sex. Humphrey (1929 *a*) recently reports that this tendency of reversal is a common feature in the larval testes of *Ambystoma*. The question, however, remains unsolved whether such a tendency has resulted from certain changes produced in the genital glands by environmental conditions, or from inherent peculiarities in the sex-differentiating agency. As pointed out in our previous papers (Cheng, 1929, 1930), certain abnormalities in the development of sex may probably be induced by genetical defects in the zygotic sex constitution, or in the embryonic mechanism of sex differentiation. Further evidence for this will be presented in a separate publication. Swingle (1917) is inclined to believe that an unequal distribution of chromosomes at the spermatocyte division may account for the appearance of hermaphroditic forms. Lloyd (1929) has pointed out that if the natural occurrence of hermaphrodite frogs is due to environmental influences, "it would be reasonable to expect, in any given frog population in which a single hermaphrodite occurs, a good percentage of frogs similarly affected, as presumably they would all have been subjected to the same influences. That this is not the case is shown by the rarity of hermaphrodites amongst frogs." The work of Goldschmidt and others on the gipsy moth has clearly shown that a great variety of intersexual conditions may result from genetical causes. Data from sex-intergrade pigs (Baker, 1925, 1929) lend support to the inheritance of a tendency to sexual abnormality. In Amphibians, intersexuality and sex reversal apparently can be produced artificially by various experimental means, such as parabiosis

(Burns, 1925; Witschi, 1927), gonad grafting (Burns, 1928; Humphrey, 1929 *b*), high temperature (Witschi, 1914, 1929*a*), nutritional disturbance (Champy, 1921) and the like. Crew (1921 *b*) and Witschi (1923) have performed breeding experiments on hermaphroditic frogs in an attempt to determine their genetical nature. The results obtained appear to indicate that those hermaphrodities are of the female type, but, as elsewhere pointed out by the author, do not show that they are genetically normal females, a significant fact which has been overlooked by all previous investigators. Until more is known about the genesis and genetics of sporadic intersexuality, it would seem premature to discuss whether the above described frog is genetically intersexual, or genetically male with an initial deficiency of sex differentiating substance, or genetically female transforming into a male.

The writer wishes to express his sincere gratitude to Prof. Peter Okkelberg under whom this research has been conducted.

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INITIATION OF DEVELOPMENT IN ARBACIA. VI. THE EFFECT OF SEA-WATER PRECIPITATES WITH SPECIAL REFERENCE TO THE NATURE OF LIPOLYSIN.[1]

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In 1910 Loeb reported that uninseminated eggs of the California sea-urchin, *Strongylocentrotus purpuratus*, form membranes while in solutions of $SrCl_2$ or $BaCl_2$. Because of the heavy precipitate of $BaSO_4$ which forms on the addition of the latter salt to sea-water, Loeb preferred to use the former. Loeb's work with these salts was done in connection with his experiments on the "fertilizing" effect of foreign blood and foreign cell extracts. Since this discovery, other workers have investigated the effects of foreign sera, treated in various ways, as agents for initiating development in echinid ova. The present writer also has made a similar study. He has likewise made a study of the effects of precipitates formed in sea-water after the addition of various substances to sea-water *alone*, in causing parthenogenetic development of *Arbacia* eggs. It is the latter study which forms the subject of the present communication. The results of the former, however, are also briefly reported. The work was begun at the Marine Biological Laboratory during the summer of 1919 and extended during several later years. Some of the precipitates were prepared not only during the summer months but also in April (three different years), in May (four different years), and in September (three different years)—*i.e.*, several weeks before the beginning and in the last days of the breeding season of *Arbacia*. In order to obtain results as far as possible beyond question, I repeated all of the experiments of previous years during the season of 1928. The data here given represent those collected during that year.

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The ox serum preparations first used were those made by my former students in the Department of Physiology of the Medical School of Howard University.

I may say at once that effects obtained by others with precipitates from sea-water containing organic matter and attributed to the presence of such matter, I have obtained with precipitates *from sea-water alone*. In what now follows, therefore, I present evidence to establish the thesis that these are hypertonic effects. It thus follows that theories of fertilization based on the effects of alleged substances, procured by treatment of sea-water containing organic or other foreign matter with various reagents, in causing parthenogenetic development of echinoderm ova, call for a careful scrutiny—if not total rejection.

The Experiments.

Loeb's discovery that $BaCl_2$ added to sea-water will cause the separation of the vitelline membrane in the uninseminated egg of *Strongylocentrotus purpuratus* may serve as our point of departure. In experiments on the uninseminated eggs of *Arbacia* I found $BaCl_2$ very toxic in the concentrations employed. I began, following Loeb, with a $\frac{3}{8}$ grammolecular solution.

$BaCl_2$.

Varying quantities of $\frac{3}{8}$ M or a 7 per cent. solution of $BaCl_2$ were added to egg suspensions. Drops of the eggs were removed from the $BaCl_2$-sea-water to 250 cc. of sea-water at half hour intervals during twelve to sixteen hours, and their development noted. During this period of time, samples of the eggs in the solutions were examined and the number of eggs with separated membranes counted. I have never found a cleaving egg either in the solution or in the sea-water to which I removed the eggs. I did find eggs with separated membranes and with thickened cortices. The eggs treated with $BaCl_2$ tend to clump in masses of the precipitate thrown down when $BaCl_2$ solution is added to sea-water, and they cytolyze in numbers. Twelve to sixteen hours after treatment with $BaCl_2$ many eggs are almost perfectly transparent, show their pigment aggregated in one mass, or form an extrusion of clear protoplasm. The eggs also frag-

ment into spheres as small as the blastomeres of a normal egg in the thirty-two cell stage. These phenomena are also observed in eggs that are kept in the $BaCl_2$ solutions.

$BaCl_2$ plus Na_2SO_4.

For the experiments on the effects of $BaCl_2$ plus Na_2SO_4 in initiating development of uninseminated *Arbacia* eggs, two methods were used to prepare the activating agent. (1) To $BaCl_2$ solution plus sea-water Na_2SO_4 was added in excess several hours after the $BaSO_4$ precipitate had been completely thrown down. The clear supernatant solution, carefully decanted, was used as the activating agent. (2) After the addition of $BaCl_2$ solution to sea-water, the clear supernatant solution above the precipitate was discarded; to the precipitate treated with M/10 HCL Na_2SO_4 was added to excess. Here again, the clear supernatant solution was used as the activating agent. As in the work with $BaCl_2$ alone, a $\frac{3}{8}$ M solution was used in varying proportions with sea-water. However, for reasons that will be made apparent beyond, in the majority of the experiments, a 7 per cent. solution of the salt was preferred. In all experiments made with this strength of $BaCl_2$, 1 part of the solution was added to 2 parts sea-water.

TABLE I.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF A 90 MINUTE EXPOSURE TO "$BaCl_2$-Na_2SO_4-SEA-WATER."

No.	1	2	3	4	5	6	7	8	9	10	11	12
Per cent. of cleavage	40	54	64	48	17	67	0	46	37	48	0	2
Per cent. of monaster and cytaster eggs	35	18	32	18	63	33	0	0	0	0	0	0
Per cent. of undifferentiated eggs	10	2	2	4	11	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	15	26	0	30	9	0	100	54	63	52	100	98
"Swimmers" (20 hours after treatment)	++	++	+	+	++	+	0	+	+	+	0	0
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0	0	0

Table I gives the results of 12 experiments on the eggs—from 12 different females—whose fertilizable condition was known by the following tests: 1, speed and strength of the sperm agglutinating power of the egg sea-water; 2, rapidity and quality of membrane separation; 3, uniformity and rate as well as per cent. of cleavage; 4, viability of the plutei. That is, before an experiment was made Nos. 1 and 2 were determined. During its course Nos. 3 and 4 were determined. Elsewhere I have discussed the importance of certain cortical reactions as criteria for optimum fertilization capacity. The reader is referred to this paper for further details (Just, 1928). I now never make an experiment with eggs before testing them to determine their cortical responses to insemination. And whenever these are optimum I find that the eggs give close to 100 per cent. cleavage at an almost uniform rate; the larvæ are always vigorous.

Table I reveals that "$BaCl_2$-Na_2SO_4-sea-water" is an efficient parthenogenetic agent. The "swimmers" were merely estimated, not counted, since the amount of cytolysis after twenty hours is so great that counts are meaningless.

Since it seemed from these experiments made by treating the supernatant "$BaCl_2$-sea-water" with excess of Na_2SO_4 that the parthenogenetic agent is Na_2SO_4, I studied the effects of this salt added in excess to sea-water. I made this experiment many times during 1928. The appended table (Table II) of twenty-two experiments reveals that Na_2SO_4 in sea-water will initiate development. In these experiments the exposures varied from 40 to 90 minutes. I therefore made eight experiments in each of which I removed the eggs from the "Na_2SO_4-sea-water" to 250 cc. of sea-water after 30, 60, 90 minutes' exposure. The results are tabulated in Table III as Lot *A* (30 minutes' exposure), Lot *B* (60 minutes'), and Lot *C* (90 minutes'). It is at once apparent that there is a great deal of variation in the response of the eggs, as measured by the per cent. of cleavage and the production of swimmers, which cannot be clearly correlated with the length of exposure. It is also apparent, however, judging by the per cent. of cytolysis, that the shorter exposure is least harmful. Indeed, this is the case. While in the "Na_2SO_4-sea-water," the eggs show membranes after ten to fifteen

TABLE II.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF 40 TO 90 MINUTES' EXPOSURE TO "Na_2SO_4-SEA-WATER."

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
Per cent. of cleavage	61	50	16	96	11	21	91	0	36	59	63	46	64	61	62	37	81	33	24	64	12	91
Per cent. of monaster and cytaster eggs	22	38	80	4	85	79	6	0	0	25	27	36	27	37	28	56	16	54	53	33	15	1
Per cent. of undifferentiated eggs	13	6	0	0	1	0	2	0	0	0	1	12	0	0	5	0	0	0	0	0	0	1
Per cent. of cytolysis 3 hours after exposure	3	6	4	0	3	0	1	100	64	16	9	6	9	2	5	7	3	13	23	3	73	7
"Swimmers" (24 hours after exposure)	+	++	+	+	+	+	+	0	+	+	+	+	+	+	+	+	+	0	0	+	0	++
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

minutes' exposure. Double this length of time, twenty to thirty minutes, is undoubtedly near the optimum exposure. The cleavages exhibited and the swimming forms produced by eggs so treated far more closely resemble those in the normal development of fertilized eggs. On the whole I consider Na_2SO_4 in sea-water an effective parthenogenetic agent.

TABLE III.

EFFECT OF 30 (LOT *A*), 60 (LOT *B*), AND 90 (LOT *C*) MINUTES' EXPOSURE TO "Na_2SO_4-SEA-WATER."

Lot A.

No.	1	2	3	4	5	6	7	8
Per cent. of cleavage	47	44	63	55	51	25	66	48
Per cent. of monaster and cytaster eggs	47	47	37	43	44	69	34	48
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	6	6	0	2	5	6	0	4
"Swimmers" (20 hours later)	+++	+++	++	+	++	+	+++	++++

Lot B.

Per cent. of cleavage	54	89	92	88	37	65	57	59
Per cent. of monaster and cytaster eggs	36	6	4	10	41	23	25	39
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	10	5	4	2	22	12	8	3
"Swimmers" (20 hours later)	0	++	+++	++	0	+	+	+

Lot C.

Per cent. of cleavage	59	85	87	86	15	67	56	14
Per cent. of monaster and cytaster eggs	30	8	9	10	26	18	12	80
Per cent. of undifferentiated eggs	0	0	0	0	0	0	0	0
Per cent. of cytolyzed eggs 3 hours after exposure	11	7	4	4	59	15	32	6
"Swimmers" (20 hours later)	0	+	++	++	0	+	++	+
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0

The Precipitate Obtained from "Na_2SO_4-Sea-water" after the Addition of Acetone.

If to the supernatant solution drawn from "Na_2SO_4-sea-water" acetone be added, a voluminous precipitate forms. This precipitate dissolved in sea-water is a parthenogenetic agent. Sixteen experiments are cited.

In six experiments the procedure was as follows: To 200 cc. of sea-water Na_2SO_4 was added to excess. 60 cc. of the clear supernatant solution was carefully decanted and to it 300 cc. of acetone added. The voluminous precipitate was then thoroughly dried and dissolved in 70 cc. of sea-water.

1 cc. of eggs was removed from the eggs (of a single female), which had been allowed to settle in 250 cc. of sea-water, to 10 cc. of sea-water in which the acetone precipitate was dissolved. The results are tabulated in Table IV.

Table IV.

Effect on Uninseminated *Arbacia* Eggs of a 60 Minutes' Exposure to a "Na_2SO_4-Sea-water" Acetone Precipitate Dissolved in Sea-water.

No.	1	2	3	4	5	6
Cleavage	27	13	92	76	71	64
Monasters and cytasters	0	0	1	0	0	10
Undifferentiated	0	0	0	0	0	0
Cytolysis	73	87	7	24	29	26
Swimmers after 20 hours	0	0	0	0	0	0
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0

Ten other experiments were made in this wise:

To 200 cc. of sea-water, Na_2SO_4 was added to excess. 60 cc. of the clear supernatant solution was carefully decanted and to it 250 cc. of acetone were added. The heavy precipitate was then thoroughly dried for 48 hours and dissolved in 200 cc. of sea-water. As before 1 cc. of eggs (of a single female) was removed from 250 cc. of sea-water to 20 cc. of sea-water in which the acetone precipitate was dissolved. At 30, 60, and 90 minutes later samples of the eggs were removed to 250 cc. of sea-water. The results are tabulated—Lot *A* is the 30 minutes' exposure; Lot *B*, the 60 minutes'; and Lot *C*. the 90 minutes'—in Table V.

TABLE V.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF A 30, 60, AND 90 MINUTES' EXPOSURE TO "Na_2SO_4-SEA-WATER" ACETONE PRECIPITATE DISSOLVED IN SEA-WATER.

Lot A. 30 minutes' exposure.

No.	1	2	3	4	5	6	7	8	9	10
Cleavage	24	30	1	31	9	74	9	6	22	4
Monasters and cytasters	73	68	97	68	81	25	87	91	74	89
Undifferentiated	2	2	0	0	10	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	1	0	2	1	0	1	4	3	4	7
"Swimmers" 20 hours later	0	0	0	0	0	0	0	0	0	0

Lot B. 60 minutes' exposure.

Cleavage	62	86	36	24	72	79	94	90	76	56
Monasters and cytasters	32	4	53	70	22	7	2	4	18	7
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	6	10	11	2	6	4	4	6	6	37
"Swimmers" 20 hours later										

Lot C. 90 minutes' exposure.

Cleavage	82	38	10	32	52	15	24	15	17	12
Monasters and cytasters	12	39	2	58	7	1	1	0	2	1
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	6	23	88	10	36	84	75	85	81	87
"Swimmers" 20 hours later	++ +++	+	0	++	++	+++ ++	++	+	+++	+++
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0

THE PRECIPITATE OBTAINED FROM "$BaCl_2$-Na_2SO_4-SEA-WATER" AFTER THE ADDITION OF ACETONE.

Experiments were also made with the precipitate obtained by adding acetone to the "$BaCl_2$-Na_2SO_4-sea-water." The first method of preparation employed was as follows:

200 cc. of clean filtered sea-water and 100 cc. of 7 per cent. $BaCl_2$ were kept for two hours with frequent stirring at 37° C. The suspension was then centrifuged and the supernatant solution discarded. The precipitate, after washing with $BaCl_2$, was

treated with N/10 HCl. To this was added Na_2SO_4 in excess and the mixture centrifuged. The clear supernatant solution was decanted and to it were added four volumes of acetone. This was then filtered and the precipitate washed with three changes each of absolute alcohol and of ether. It was dried for 48 hours before the addition of sea-water.

The second method employed follows:

TABLE VI.

EFFECT ON UNINSEMINATED *Arbacia* EGGS OF A 30 AND 60 MINUTES' EXPOSURE TO "$BaCl_2$-Na_2SO_4-SEA-WATER" ACETONE PRECIPITATE DISSOLVED IN SEA-WATER.

Lot A. 30 minutes' exposure.

No.	1	2	3	4	5	6	7	8	9	10
Cleavage	49	59	82	62	66	92	85	26	66	77
Monasters and cytasters	37	22	10	11	9	7	8	40	26	10
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	14	19	8	27	25	1	7	34	8	13
"Swimmers" 20 hours later	+	+	+++	+	++	+++ +	+	+	+	++

Lot B. 60 minutes' exposure.

Cleavage	20	15	59	10	5	91	35	35	34	40
Monasters and cytasters	0	0	0	0	0	1	0	2	2	0
Undifferentiated	0	0	0	0	0	0	0	0	0	0
Cytolysis 2 hours after return to sea-water	80	85	41	90	95	8	65	63	64	60
"Swimmers" 20 hours later	+	+	0	+	+	+	+	0	0	+
Controls: uninseminated eggs in sea-water. Per cent. of membranes, cleavage, and larvæ	0	0	0	0	0	0	0	0	0	0

To 200 cc. of clean filtered sea-water 100 cc. of a 7 per cent. solution of $BaCl_2$ were added. After 24 hours the clear supernatant solution was decanted and Na_2SO_4 added to excess. This in turn was decanted and acetone added to the clear solution. The ten experiments, given in the table appended (Table VI), were made with this method, the results of the first method being reserved for mention beyond. In these experiments eggs were removed from the solution after 30 (Lot *A*), 60 (Lot *B*), and 90 (Lot *C*) minutes to normal sea-water. In all cases, however, ninety minutes' exposure resulted in their complete

cytolysis, as revealed by careful examination two hours after their return to normal sea-water.

The Precipitate Obtained from Sea-water after the Addition of Acetone.

If acetone be added to clean filtered sea-water in the proportions, 1000 cc. of acetone to 250 cc. of sea-water, a flocculent precipitate is formed. This precipitate after thorough drying for twenty-four or more hours when suspended in 20 cc. of sea-water has feeble parthenogenetic power. For example, in ten experiments one drop of eggs was exposed to the action in 2 cc. of the acetone-precipitate-sea-water in tightly stoppered vials for two hours with the following results:

No. 1—0 per cent. cleavage; 31 per cent. cytolysis; 69 per cent. intact.
No. 2—2 per cent. cleavage; 84 per cent. cytolysis; 14 per cent. intact.
No. 3—0 per cent. cleavage; 38 per cent. monasters; 62 per cent. intact.
No. 4..... 12 per cent. cytolysis; 88 per cent. intact.
No. 5—2 per cent. cleavage; ..... 98 per cent. intact.
No. 6..... 50 per cent. cytolysis; 50 per cent. intact.
No. 7..... 10 per cent. cytolysis; 90 per cent. intact.
No. 8—1 per cent. cleavage; ..... 99 per cent. intact.
No. 9..... 100 per cent. intact.
No. 10—1 per cent. cleavage; 99 per cent. monasters.

In other experiments, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 cc. of sea-water were added to the dried acetone-sea-water-precipitate. From these mixtures 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cc. were taken as the parthenogenetic agent. The action of the acetone precipitate obtained from normal sea-water is at best feeble. But I have never failed to obtain the precipitate when acetone is added to sea-water; six different "C. P." preparations of acetone gave the same results. And at Naples, I obtained this precipitate with the use of ordinary "commercial" acetone.

The Precipitate Obtained from Sea-water after the Addition of 95 per cent. of Absolute Alcohol.

Beyond demonstrating that on addition of 95 per cent. of absolute alcohol to sea-water a flocculent precipitate is formed, I have not proceeded farther because of the high cost of alcohol.

I nevertheless attach importance to this fact. During the last six seasons at Woods Hole I have repeatedly demonstrated to fellow workers the action of both acetone and alcohol in throwing down a precipitate when either is added to sea-water.

Thrombin from the Serum of Ox Blood.

Thrombin from the serum of ox blood prepared either by the Schmidt or the Howell method when thoroughly dried exerts a feeble effect on the uninseminated eggs of *Arbacia*. This effect is revealed by cortical changes in the eggs and the production of a small per cent. of monasters, cleavage, and abnormal swimmers. Though the experiments were made during several years, I do not regard them worthy of more than passing notice at this point.

Hirudin and Curare.

A sample of "pre-war" hirudin in sea-water caused slight cortical changes in uninseminated *Arbacia* eggs with a small per cent. of cleavages and less than 0.1 per cent. swimmers. Suspensions of curare in sea-water were found less effective.

Discussion.

In 1918, Woodward reporting certain results obtained with precipitates from the "egg-sea-water" of *Arbacia* after its treatment with certain reagents, claimed the discovery of a parthenogenetic agent elaborated by the uninseminated eggs themselves and escaping into the surrounding sea-water. This agent Woodward named lipolysin. Her method of preparation is given in the following quotation.

"Precipitation of a lipolysin. Since there were strong indications of the presence of lipase and perhaps other enzymes, I adapted a method used by Robertson ('12) to obtain an enzyme from blood serum. Eight volumes of fertilizin and four volumes of 7 per cent. $BaCl_2$ were kept at 37° C. for an hour or more and stirred frequently. The mixture was then centrifuged and the filtrate discarded. The precipitate was washed several times with $BaCl_2$ and then treated with N/10 HCl. To this solution was added Na_2SO_4 in excess to precipitate the barium, and the mixture allowed to stand overnight. The liquid was then cen-

trifuged and to the clear fluid were added four to five volumes of acetone, which caused a heavy flocculent precipitate. This was filtered, and the precipitate was washed several times with absolute alcohol and ether, and then dried for thirty-six hours or more over H_2SO_4. The resulting powder dissolves readily in both sea-water and distilled water. It should be added that this precipitate was invariably obtained with good fertilizin and freshly distilled acetone during the summer of 1915 and until the middle of July, 1916. It was never obtained by adding acetone to sea-water. By the middle of 1916, all of the recently purchased acetone had been used up, and recourse was necessary to some purchased from Kahlbaum in 1912. This was yellowish in color, slightly acid to litmus, and differed slightly in odor from the fresh. With this acetone, no precipitate was obtained excepting after adding NaOH, and even then only in traces." (Woodward, 1918, pages 475–476.)

Using normal sea-water instead of "egg sea-water," I have followed Woodward's method exactly. On obtaining positive results—even better than those she recorded—I next analyzed separately the parthenogenetic action of each reagent she employed. The experiments detailed above constitute the evidence for the statement that the use of egg-sea-water for the precipitation of lipolysin is wholly superfluous; this "lipolysin" is present in normal sea-water after its treatment with any one or all of the reagents in Woodward's method. The alleged "lipolysin" is merely hypertonic sea-water. Let us summarize the results which I have given above:

1. $BaCl_2$ as Loeb showed years ago for eggs of *S. purpuratus* has a feeble parthenogenetic effect. This is likewise true for the uninseminated eggs of *Arbacia*.

2. Either the precipitate formed after the addition of $BaCl_2$ to normal sea-water when treated, as Woodward treated "egg-sea-water," with Na_2SO_4 or the solution above the $BaSO_4$ precipitate in sea-water on the addition of Na_2SO_4 to excess is a good parthenogenetic agent.

3. Na_2SO_4 added to normal sea-water in excess is an effective parthenogenetic agent.

4. The precipitate obtained after the addition of acetone

to "$BaCl_2$-sea-water" or to "$BaCl_2$-Na_2SO_4-sea-water" brings about parthenogenetic development. The washed, HCl-treated precipitate from normal sea-water thrown down by $BaCl_2$ after the addition of Na_2SO_4 to excess has a similar effect. It did not seem worthwhile to give above the details of this latter finding.

Consideration of these four lines of evidence leads one to the conclusion that the effects are due to hypertonicity and not to the presence of a lipolysin. Simply the results with Na_2SO_4 in normal sea-water acting alone are fatal to Woodward's theory of the initiation of development. Moreover, the experiments made to learn the effects of the precipitate formed on the addition of acetone to normal sea-water, if the four lines of evidence summarized were wanting, would go a long way toward discrediting Woodward's theory. She says that she never obtained a precipitate by adding acetone to sea-water. With both "c.p." acetone of all manufacture available and commercial acetone, as well as with ninety-five per cent. and absolute alcohol, I have never failed to get a voluminous flocculent precipitate from normal sea-water. Even if this precipitate had no effects on the uninseminated eggs, the fact that it forms under these conditions is alone a serious obstacle to the acceptance of Woodward's work.

Now I have obtained precipitates from "Na_2SO_4-sea-water" and "$BaCl_2$-Na_2SO_4-sea-water" after the addition of acetone as early as April, weeks before the breeding season of *Arbacia* begins. Again, in the last days of September, when ripe eggs of *Arbacia* are scarce or indeed unobtainable, have I obtained the precipitate. Surely, there could scarcely be available during these months any lipolysin in the normal sea-water around Woods Hole!

The objections against the lipolysin theory thus appear, to me at least, far too serious to warrant its acceptance.[1] More-

[1] It is extremely doubtful, for example, that according to her own description, Miss Woodward ever observed agglutination of *Asterias* sperm by *Asterias* egg-water. According to her description, if a drop of egg-water was "injected under a cover-glass into a drop of sperm suspension, the sperm would gather into small irregular angular clusters of six to eighteen and remain agglutinated for a number of seconds." This is certainly not comparable to agglutination in *Arbacia*, *Echina-*

over, built in a measure on Glaser's theory of auto-parthenogenesis, which itself is open to grave suspicion (see Just, '28 *b*), it can only deserve a hearing by the establishment of wholesale errors in this present communication.

I have elsewhere pointed out grave defects in the lipolysin theory: logically, it does not and cannot explain either experimental parthenogenesis or fertilization—even if its experimental basis be correct. More, a mere superficial reading of Woodward's paper reveals it to the reader as a mass of contradictions. Both on grounds of experiment and on grounds of logic, therefore, the lipolysin theory is lacking.

The lipolysin theory thus belongs in the same category with Robertson's ovocytase. I believe that thrombin from the serum of ox blood, prepared by either the method of Schmidt or that of Howell is as effective as Robertson's so-called ovocytase. That it would be dangerous to theorize on this point, one may conclude from the effect of hirudin. Thrombin *per se* has no particular virtue if it be true that hirudin likewise—like a host of foreign substances—effects changes in the egg of *Arbacia*. Robertson's work too generally has been allowed to go unchallenged. It is work like this that needlessly cumbers progress in the field of fertilization.

The purpose of this first work (1914) was to study the effect of thrombin as an activating agent. I was led to this work

rachnius, or *Nereis*. Lillie has repeatedly failed to obtain agglutination in *Asterias*. Glaser claims to have observed agglutination of starfish sperm but gives no details of his observations. On the other hand, using normally shed eggs and sperm over a period of years, I have yet to observe agglutination of *Asterias* sperm by *Asterias* egg-sea-water.

It should be noted, however, that absence of a sperm agglutinin in *Asterias* egg-water is of no consequence for Miss Woodward's theory since she claims to have demonstrated that the sperm agglutinin is distinct from "Lipolysin" (478, 480, 487). It is the "lipolysin" alone that plays the leading rôle in the initiation of development. It would thus seem that any criticism of the lipolysin theory based on the absence of sperm agglutinin in *Asterias* egg-water is beside the point. But this is not wholly true, it would seem from Miss Woodward's own statements: "Lipolysin" is a lipolytic enzyme, possessed of parthenogenetic power, while the other separate entity, the sperm agglutinin, lacks it. Says Miss Woodward: "If the agglutinin is an enzyme, its nature is not yet known" (page 479), though the effect of x-radiation suggests this. One is thus led to infer that the effect of the x-radiation is on the enzyme, "lipolysin," which is present but not identical with the agglutinin. Its mere presence, however, is sufficient to make the agglutinin behave like an enzyme when subjected to x-radiation.

through reasoning somewhat like this: The fertilization reaction might be comparable to the action of thrombin in the coagulation of blood. There might thus be two possible modes of interpreting the reaction in a manner similar to two of the then existing theories of blood coagulation: First, the egg produces a substance which with calcium in the sea-water forms a substance; and it is this substance with which the sperm unites to bring about fertilization. Or, fertilization results when a substance, comparable to blood thrombin, is in excess of another substance, comparable to antithrombin, and with which the sperm unites.

Woodward's lipolysin theory of fertilization more closely resembles the latter scheme. Unfortunately, it seems to me, her work is open to question. Subsequent work convinced me that any notion of my own concerning the analogy between blood coagulation and fertilization is only superficial. The effects of thrombin, were they to induce one hundred per cent. development, could scarcely give support to this notion. And if they did, the effects of hirudin would outweigh it.

On the other hand, Lillie's fertilizin theory does resemble the following scheme for the explanation of the blood coagulation:

Cellular elements → thrombokinase
Thrombokinase + calcium + thrombogen = thrombin
Thrombin + fibrinogen = fibrinogen.

That is, according to the fertilizin theory, the fertilization-reaction might run thus:

Cellular elements (the eggs) → fertilizinkinase
Fertilizinkinase + calcium = fertilizin
Fertilizin + sperm-receptor = fertilization.

But this scheme at best is only a crude and superficial analogy. Moreover, after I had made experiments on the effects of oxalated sea-water (1915) and on the fertilization capacity of eggs in various salt solutions (referred to in Lillie and Just, 1924) I abandoned this notion. Even if the scheme for explaining blood coagulation given above were correct, it would be hazardous to compare this phenomenon with fertilization. Lillie's original statement of his theory thus remains the best working hypothesis for the analysis of fertilization. Fertilization is a

simple two-body reaction—a reaction between an egg substance, fertilizin, and the spermatozoön.

I have repeatedly emphasized the necessity of analyzing the fertilization-reaction in terms of the behavior of the gametes themselves in the presence of each other. The work on experimental parthenogenesis makes up a brilliant chapter in modern biology; but experimental parthenogenesis is more significant for the analysis of cell division than for that of the reaction between gametes. Agents of experimental parthenogenesis from the very nature of the case can at best never fully bring about in the egg those changes initiated by insemination. Consider for a moment the results, which I have recently summarized (1928 *a*), obtained with various agents that induce membrane separation in *Arbacia* eggs. These agents, if they do not call forth the optimum response by the eggs, do too little, and the eggs show no change; or they do too much, and the eggs cytolyze. What is more important, however, is that eggs with membranes separated by the action of experimental agents *are not in the same condition as eggs with membranes induced by insemination.* The basis for this statement is the fact that eggs with membranes separated by such agents do not respond with the formation of extra-ovates when placed in distilled water; on the other hand, eggs with membranes separated after insemination do form extra-ovates. The majority of the agents used for experimental membrane separation are presumably effective over the whole surface; the spermatozoön is rapidly effective within an exceedingly narrow cortical *locale.* Again, with the best methods for experimental parthenogenesis eggs often fail to respond though they are capable of fertilization. Finally, the per cent., normality, and viability of the development induced by experimental agents under the best conditions frequently fall short of those which result from insemination.

If these statements be true, it must follow that physico-chemical explanations of fertilization though based on refined and technically excellent physico-chemical work on experimental parthenogenesis demand careful criticism. It is my judgment that unless agents of experimental parthenogenesis exactly duplicate the morphology (or biology) of fertilization in every egg exposed, the interpretations by the modernistic school of physico-,

chemico-, and mathematico-biologists of fertilization in terms of experimental parthenogenesis rest on insecure grounds. For example, the measurement of the oxygen consumption, the CO_2 output, the viscosity changes, and the whole category of physico-chemical changes exhibited by eggs with membranes separated after treatment with agents that cause the eggs to go no farther in their development are exceedingly important. Thus, also, similar measurements on eggs so treated (by hypertonic sea-water, for instance) that they develop without the cortical responses typical of inseminated eggs. But in neither case, however, are we dealing with responses exactly similar to those brought about by insemination. The present status of the fertilization problem, therefore, demands a rigorous *biological* analysis of the fertilization-reaction itself, before we can venture a physico-chemical approach to our problem. In addition, finally, we must appreciate the limited applications of the work on experimental parthenogenesis to fertilization.

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EFFECTS OF LOW TEMPERATURE ON FERTILIZATION AND DEVELOPMENT IN THE EGG OF *PLATYNEREIS MEGALOPS*.[1]

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Having previously learned that low temperature induces a per cent. of the eggs of both *Nereis* and *Platynereis* to develop after insemination without the extrusion of the polar bodies, I ran some additional experiments on the eggs of the latter during the summer of 1927. This I did because the nuclear phenomena show so clearly in the living egg. I therefore hoped to be able to isolate, just before first cleavage, eggs with quintuple haploid nuclei for the purpose of rearing them to sexual maturity. Eggs of *Platynereis* were chosen because I have had a greater degree of success in rearing them than those of *Nereis*. The method of exposure to cold was preferred to that of ultra-violet radiation; since normal eggs obtained only after copulation of the worms are already inseminated and since exposure to ultra-violet up to five minutes is without effect on the eggs in a virgin female, uninseminated eggs cannot be subjected to radiation in sea-water as is the case with those of *Nereis*. The exposure of dry eggs, which are capable of normal fertilization and development, to ultra-violet radiation has not yet been undertaken.

THE EXPERIMENTS.

Two series of experiments were made: one on the effects of low temperature on uninseminated eggs in the females; and one on eggs normally laid.

Forty-eight females were placed in the cold chamber at a temperature of 3.5° C. After ten hours at this temperature they were removed. As soon as the water containing them reached the temperature of the sea-water in the laboratory and at intervals

[1] From the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoölogy, Howard University, Washington, D. C.

thereafter they were allowed to copulate each with one male from those kept over-night in normal sea-water. The per cent. of cleavage bore no constant relation to the time of copulation and egg-laying as the following tabulation reveals:

No.	Time of Egg-laying.	Per cent. No Cleavage.	Per cent. Cleavage.
1	11:05 A.M.	100	0
2	11:10	100	0
3	11:11	44	56
4	11:12.5	100	0
5	11:15.5	100	0
6	11:17.5	97	3
7	11:22	100	0
8	11:24.5	100	0
9	11:26	74	26
10	11:28	100	0
11	11:32.5	100	0
12	11:38.5	91	9
13	11:41	50	50
14	11:44.5	100	0
15	11:53	18	82
16	11:54	68	32
17	11:55.5	100	0
18	11:58.5	74	26
19	12:02 P.M.	100	0
20	12:12	99	1
21	12:14	34	66
22	12:15	60	40
23	12:16.5	64	36
24	12:18	92	8
25	12:20	74	26
26	12:23	88	12
27	12:30	68	32
28	12:30	100	0
29	12:31	96	4
30	12:35.5	100	0
31	2:25.5	100	0
32	2:27	99	1
33	2:31.5	100	0
34	2:34.5	100	0
35	2:36	26	74
36	2:37	42	58
37	2:40	42	58
38	2:41	92	8
39	2:42.5	96	4
40	2:46	100	0
41	2:47	62	38
42	2:48.5	94	6
43	2:54	56	44
44	2:55	100	0

It would be erroneous to attribute these results entirely to the effects of low temperature for the reason that *Platynereis*, especially the males, are not good laboratory animals. That is,

under laboratory conditions ideal for *Nereis*, for example, they early suffer a loss of vitality since animals kept over-night at room temperature never give normal fertilization and development.

The other series of observations was made on the development of eggs exposed to low temperature after egg-laying.

Twenty-five female *Platynereis* were allowed to copulate immediately after capture, each with a different male. Three minutes after laying, each lot of eggs was placed in the cold chamber at a temperature of 3.5° C. The next day (ten hours after egg-laying) the eggs were brought from the cold chamber and allowed to return gradually to room temperature without changing the sea-water. The results obtained are recorded as follows:

Female No.	Time of Egg-laying.	Per cent. Cleavage.	Per cent. "Swimmers."
1.....	10:39 P.M.	50	4
2.....	10:40	6	2
3.....	10:41	74	6
4.....	10:43.75	18	4
5.....	10:41.75	88	50
6.....	10:44	60	14
7.....	10:43.5	16	0
8.....	10:40.75	78	14
9.....	10:41.5	92	28
10.....	10:43.5	78	14
11.....	10:43	14	2
12.....	10:43.5	84	70
13.....	10:41.7	74	16
14.....	10:40.5	86	46
15.....	10:41	82	28
16.....	10:43	90	32
17.....	10:43.6	64	20
18.....	10:46	68	32
19.....	10:45	74	24
20.....	10:45	98	12
21.....	10:45	98	16
22.....	10:47	84	28

Because the per cent. of first cleavage in many cases is difficult to ascertain, it is best to make counts when the majority of cells are in the eight cell stage; later, it is still easier to make the counts. The oil drops, especially in those eggs that fail to cleave, are abnormally reduced in number; many eggs show the oil drops coalesced into one. Many cells that do not show cleavage show nuclear divisions, even in cases where the oil

drops are normal in number; such eggs differentiate as far as the swimming stage without cleavage.

Discussion.

The action of low temperature on eggs is by no means simple. This is especially true for the egg of *Platynereis*. However one of its effects on this egg is to inhibit the extrusion of polar bodies with or without subsequent normal development. The retention of the polar bodies within the cell brings about the formation of four maternal haploid nuclei with one, two, three, or all of which the sperm nucleus unites. This fact has been ascertained by study of the living egg; the phenomenon was (during 1926 and 1927) demonstrated to several fellow workers. Where, however, as in most cases, development is abnormal or fails we must not conclude that the results are due to polar body retention and the consequent presence of female nuclei above the orthodox single zygote nucleus.

In addition, the effect of low temperature presumably is not localized to any one area of the cortex; the whole cortex is affected. If this be true, it follows that the production of quintuple zygote nuclei is not due to localized injury. *Nereis* eggs inseminated after ultra-violet radiation reveal a localized cortical injury which endures through the stages of development including the trochophore. Ultra-violet radiation also causes alteration of the polarity of the egg. It may be that further comparative study of the effects of low temperature and of ultra-violet radiation on the eggs of these nereids may give us valuable information concerning the rôle of the cortex in determining polarity.

HYDRATION AND DEHYDRATION IN THE LIVING CELL. II. FERTILIZATION OF EGGS OF *ARBACIA* IN DILUTE SEA-WATER.[1]

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Several years ago I made observations on the fertilization of eggs of *Echinarachnius parma* in dilute sea-water (Just, '23). These observations have now been extended to include the eggs of several forms (Just, '28). During the summer of 1927, working at the Marine Biological Laboratory, Woods Hole, Mass., I repeated some older observations on the fertilization capacity of eggs of *Arbacia* in dilute sea-water. These observations early established several interesting facts, among them: first, that in the greatest dilutions in which fertilization is possible, the time of first cleavage is delayed; secondly, that fertilization capacity in these dilutions depends upon the concentration of the egg suspensions—*i.e.*, it is greatest when the suspension is attenuated; thirdly, the agglutination of sperm is possible in dilute sea-water; and finally, that fertilization is possible in greater dilution than that in which eggs of *Echinarachnius* are capable of fertilization. The present communication, however, deals with the last-named finding only. The action of the dilutions employed is wholly reversible: eggs exposed to them for hours on return to normal sea-water fertilize and develop normally.

THE EXPERIMENTS.

Eggs of *Arbacia* fertilize readily in dilutions of 95, 90, 85, and 80 parts of sea-water plus 5, 10, 15, and 20 parts respectively of distilled water, as repeated experiments show. (Cf. Medes.) On ascertaining this fact I did not proceed farther since the object of my experiments was to learn the greatest degree of hypo-

[1] From the Marine Biological Laboratory, Woods Hole, Mass., and the Department of Zoölogy, Howard University, Washington, D. C.

tonicity in which fertilization is possible. I therefore made a large number of experiments on the fertilization of eggs in the dilutions 75, 70, and 66⅔ parts sea-water plus 25, 30, and 33⅓ parts, respectively, of distilled water. The last named dilution is close to the limit for fertilization. A protocol of a typical experiment with 75 and 70 per cent. sea-water follows:

July 7, 1927. 8:15 A.M. 3 females opened. Eggs from each to 250 cc. of sea-water. Each lot of eggs washed at 8:35, 9:08, and 9:16 A.M. At 10:10 A.M. eggs tested for fertilization capacity and the best lot retained. At 10:20 A.M., 3 graduates prepared as follows: No. 1, 100 cc. of sea-water plus 0 cc. of distilled water; No. 2, 75 cc. of sea-water plus 25 cc. of distilled water; No. 3, 70 cc. of sea-water plus 30 cc. of distilled water. At 10:28 A.M., 1 cc. of eggs added to each graduate. At 11:08 A.M., each lot of eggs poured into finger bowls and inseminated in the solutions.

12:02 P.M., first cleavage in Nos. 1 and 2; cleavage is indicated in some eggs of No. 3.

1:48 P.M., No. 1 (inseminated in sea-water), 98 per cent. cleavage—8- and 16-cell stages.

No. 2 (inseminated in 75 cc. of sea-water plus 25 cc. of distilled water), 90 per cent. cleavage.

No. 3 (inseminated in 70 cc. of sea-water plus 30 cc. of distilled water), 75 per cent. cleavage. Cleavages irregular and arrested; mostly 4- and 8-cell stages with some 3-cell stages.

6:45 P.M. All 3 dishes show good late cleavage stages. One swimming blastula in No. 1.

8:40 P.M. Normal embryos (No. 1) are swimming.

8:50 P.M. Very few "swimmers" in No. 2. No "swimmers" in No. 3.

9:47 P.M. All swimming in No. 2. About 3 per cent. swimming in No. 3; others, except those not fertilized, still in their membranes.

July 8, 7:30 A.M. Normally inseminated (No. 1) are now plutei. No. 2—50 per cent. are top swimming gastrulæ; 50 per cent. are rotating blastulæ on bottom of dish. No. 3. Great majority are abnormal blastulæ on bottom of dish away from the light; those that are suspended, early but extremely active gastrulæ.

Subsequently throughout the breeding season of *Arbacia* this experiment was repeated with the difference that greater dilutions of sea-water were used. For example, on July 8 in ten experiments with 66⅔ per cent. sea-water, the average per cent. of cleavage was 44. Of the eggs that failed to cleave, 18 per cent. showed separated membranes. In all these experiments with 70 and 66⅔ per cent. sea-water first cleavage took place later than in eggs inseminated in sea-water. Also, when these experiments were repeated daily for the next five days, it was discovered that a higher per cent. of cleavage was obtained in a given volume of dilute sea-water if only a few eggs were used. Experiments subsequently were therefore made always with 150 cc. of sea-water or given dilution to which was added *one drop* of eggs. The following is typical of a group of eight experiments:

July 16, 9:10 A.M. Each lot of eggs from nine females washed four times in 250 cc. of sea-water. Samples from each lot when tested at 11:00 A.M. show optimum fertilization reaction. 11:08 A.M., one drop of eggs from each of the nine lots added to 9 dishes containing each 150 cc. of sea-water (Series I).

Similarly one drop of eggs from each lot added to each of 9 dishes containing 100 cc. of sea-water plus 50 cc. of distilled water (Series II).

12:08 P.M., eggs of both series inseminated.

12:10–12:14 P.M. These eggs in normal sea-water show 98 to 100 per cent. beautiful membranes.

12:15 P.M., eggs of Series II (in 66⅔ per cent. sea-water) show following with respect to membranes:

No. 1—100 per cent. full membranes.

No. 2—2 per cent. full membranes, 9 per cent. tight membranes.

No. 3—50 per cent. full, 50 per cent. tight membranes.

No. 4—100 per cent. full membranes.

No. 5—94 per cent. full membranes, 4 per cent. tight membranes, 2 per cent. cytolysis.

No. 6—93 per cent. full membranes but thin; 7 per cent. tight membranes.

No. 8—98 per cent. tight membranes, 2 per cent. no fertilization.

No. 9—97 per cent. full membranes, 1 per cent. cytolyzed, and 2 per cent. fused eggs—tight membranes ruptured in each at point of fusion.

1:00 to 1:10 P.M. Samples of each of the nine lots inseminated in sea-water (Series I) show first cleavage—98 to 100 per cent.

1:35–1:45 P.M., these eggs in dilute sea-water show cleavage as follows:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	70	65	33	64	89	30	45	43	42

Eggs of Series I are now beautiful perfectly normal top "swimmers."

Quite frequently as in the experiment now to be cited eggs were allowed to stand for several hours in sea-water. At the time of transfer to the dilution an equal bulk of eggs was placed in equivalent volumes of sea-water.

For example:

July 15, 3:40 P.M., to each of 9 dishes containing 100 cc. of sea-water plus 50 cc. of distilled water is added one drop of eggs that have been in sea-water since 9:10 A.M. (6½ hours). Eggs are similarly added to 9 dishes containing 150 cc. of sea-water.

4:45 P.M., both series of eggs are inseminated.

5:45–5:55 P.M. Eggs inseminated in sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	98	99	97	98	94	96	89	78	86

6:00 P.M. No cleavages among eggs in the solution.

6:30 P.M. Some eggs show cleavage beginning.

6:40 P.M. Eggs in 66⅔ per cent. sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of cleavage	24	35	40	30	52	11	36	27	49

Whereas, however, on the next day (9:10 A.M.) the eggs inseminated in sea-water show percentages of gastrulæ almost identical to those for cleavage, these eggs in the 66⅔ per cent. sea-water give the following:

No.	1	2	3	4	5	6	7	8	9
Per cent. of blastulae	32	38	1	2	50	0	30	0	17

The counts did not include intact unfertilized eggs which means therefore that eggs if fertilized either developed or cytolyzed. Eggs in No. 9 showed many microblastulæ and twins.

Discussion.

The reversible effects of dilute sea-water or of other hypotonic solutions on the uninseminated echinid egg are too generally known to warrant lengthy discussion. That *Arbacia* eggs are capable of fertilization and development *in* a given dilution of sea-water would seem further to indicate that such dilution is not deleterious. This statement must not be taken to mean that the effects of a still greater dilution which prohibits fertilization are irreversible. However, claims that a given solution produces a reversible effect would be greatly strengthened by the simple procedure of testing the fertilization capacity of eggs which on removal from the solution have come into equilibrium with the normal sea-water.

The interpretations of the effects of hypotonic solutions on echinid ova—those of *Arbacia*, for example—are largely in terms of osmotic pressure. Now I have found, as others previously, for several ova, that sea-water of a dilution which permits fertilization the effects of which are reversible brings about the imbibition of water by the eggs while in the dilution; on their return to normal sea-water, *they show vacuoles in the cytoplasm which migrating to the egg surface disappear*. The uninseminated egg of *Arbacia* is not the most favorable object for the study of this phenomenon. Nevertheless, like the egg of *Nereis* (Just, '26), it does exhibit it. A theory of the permeability of the *Arbacia* egg to water should consider this phenomenon.

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THE TOXICITY OF MONOVALENT AND DIVALENT CATIONS FOR SEA URCHIN EGGS.[1]

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The literature describing the action of salts on animal protoplasm (1–18), plant protoplasm (18–21), special physical systems (22–31) and the more specific case of the red blood cell seems to show that the toxicity of electrolytes is not of the same order for all types of cells. This fact renders it probable that protoplasm is not necessarily a "stuff" of uniform reactivity regardless of its origin.

"Salt toxicity" is probably the composite picture of many individual reactions. Salts have the most variant actions on lipoids, soaps, proteins, etc., and since protoplasm is made up of complex mixtures of these special moieties it is difficult to conceive in our present state of knowledge how we can ever predict "toxicity." This conception seems to have been overlooked in much of the investigative work concerning the toxicity of salts especially for highly differentiated organisms. Thus sodium might be very toxic for one tissue while nontoxic for another tissue and our term "toxicity," when applied to differentiated organisms, then becomes a miscellany of the greatest intricacy, on the one hand that of complex cellular reactivities and on the other complex cellular types.

Li and Na form one, and K, Rb, and Cs the other subdivision of the alkali metal period. If only the chemical structure and related physical properties of the reagent were to determine the physiological response of the protoplasm regardless of the protoplasmic type with which we deal we might anticipate that the relations which these metals maintain in the periodic system should be maintained in their action on protoplasm. The tendency should always be toward a grouping of Li and Na on the one hand and Rb, Cs, and K on the other. In addition Li and Mg should resemble Ca in some respects and Na in others.

[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

Many types of protoplasm respond to salts in such a way as to suggest that the specific chemical nature of the salt is, indeed, largely responsible for its reactions. However one must still appreciate the fact that the reagent electrolyte is only one half of the reacting system; the sum total of the effect we call "salt toxicity."

Although an explanation of toxicity is far from forthcoming nevertheless the data on toxicity are important in order to correlate other vistas of research on protoplasm with this complex phenomenon.

Methods.

The eggs of the Sea Urchin (*Arbacia punctulata*) were used in all the experiments. It may be stated at the outset that a possible source of error occurs in the resistance of individual eggs. However the difference observed in toxicity to the electrolytes are well beyond such experimental variations.

The eggs were removed directly from the ovaries with a pipette and placed in the isotonic solution of the purest electrolyte obtainable. Particular difficulty was experienced in obtaining a good quality $CaCl_2$. Freezing point determinations for Woods Hole sea water (G. Walden (32)) gave the following molar equivalents for the electrolytes. NaCl = 0.52, KCL = 0.53, $CaCl_2$ = 0.34, $MgCl_2$ = 0.35. The following are only approximate: Na_2HPO_4 =0.40, LiCl = 0.56, CsCl = 0.53, RbCl = 0.56.

Method 1.—In order to measure the effect of salts on protoplasm it is obvious that the salt must be in contact in a pure condition with the eggs. For instance, a mere trace of Ca or Mg profoundly modifies the toxicity of the monovalent cations. It is for this reason that it was found necessary to centrifuge the eggs three times in a solution of electrolyte to be tested adding fresh electrolyte at each centrifuging. The eggs were then transferred to Stender dishes, and 5 c.c. of the suspension removed at certain intervals of time, placed in a large quantity of sea water, washed and fertilized. After 24 hours the percentage development was estimated.

Curiously enough it is important that the eggs be taken directly from the ovaries particularly when the divalent cations are used (Heilbrunn (33)) and Page (32a). Eggs which have been washed

in sea water and allowed to stand, when placed in the electrolyte solution and centrifuged, quickly agglutinate. This has been shown by Chambers (34) to be due to the fact that $CaCl_2$ causes the jelly surrounding the egg to become very sticky after the eggs are returned to sea water whereas in KCl the jelly is simply dispersed. Due to the presence of $CaCl_2$ this adhesiveness causes the eggs to stick together so tightly that when centrifuged cytolysis occurs through mechanical tearing of the eggs. The same phenomenon occurs with NaCl but not in such a striking manner as with $CaCl_2$. Why fresh, unwashed eggs show much less tendency to clump on electrolyte treatment is difficult to say.

Method 2.[1]—Since it is possible that the relatively severe centrifugation employed in the first method might influence the results, we resorted to the following technique:

Eggs shed from the ovaries were very gently centrifuged in order to remove most of the sea water. Pyrex glass tubes 5 ft. long and ½ inch in diameter were filled with the electrolyte to be tested. The eggs were then mixed with a little of the salt solution and dropped into the top of the tube. As the eggs fell slowly through the solution a continuous washing process was in action. Convection and diffusion of the salts in the upper part of the tube into the lower part could hardly occur due to the length of the tubes and their relatively small bore. These washed eggs were then placed in large bowls of sea water, washed, and fertilized. At one and half and three hours, samples were removed and fixed in 3 drops of 20% formaldehyde to 3 c.c. of egg suspension. Counts were made in each sample, noting the number of eggs which were undivided and the number which were in each stage of division. By multiplying the numbers of two celled eggs by one, the four celled eggs by two, the eight celled by three, etc. and dividing the total number of divisions by the total number of eggs, the number of divisions per egg was determined. This figure was then corrected by the control (equalled 100%) which had been subjected to the same handling in sea waer. The temperature of the sea water varied between 20–23 degrees C. The $[H^+]$ of the electrolytes used as determined colorimetrically with the

[1] Our thanks are due Miss Kellicott for her work during this part of the investigation.

Clark and Lubs indicator series wereNa Cl = 6.3,KCl = 6.4, $CaCl_2$ = 7.6, $MgCl_2$ = 6.3.

Results.

Figure 1 shows the results of treatment of Arbacia eggs with the various chlorides employing method 1 e.g. centrifuging the eggs with the electrolytes. The curves represent the average of ten

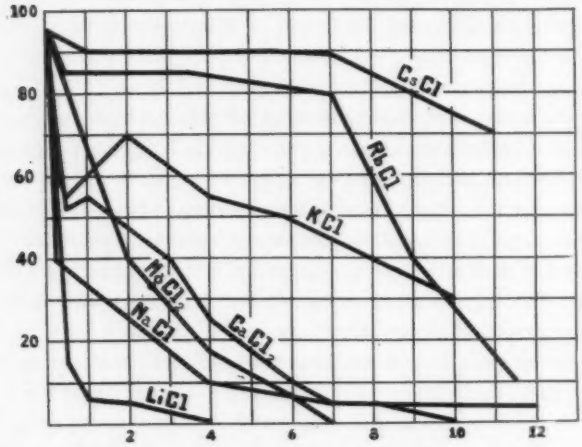

Fig. 1. Toxic effect of pure electrolytes on *Arbacia* eggs. The ordinate represents percentage of development after immersion for the period in hours in the electrolyte as represented by the abscissa. Method 1 in which centrifuging was employed to remove sea water.

experiments with each salt. The ordinates represent percentage development and the abscissæ the time of immersion in the salt solution before fertilization. It is evident from the chart that the cations can be arranged in the following toxicity series.

$$Li > Na > Mg, Ca > K > Rb > Cs.$$

Figures 2 and 3 show graphically the results of the average of nine experiments in which the divisions per egg corrected by the control is plotted against the time in the electrolyte. In Fig. 2 the eggs were allowed to develop for one and one-half hours after insemination and in Fig. 3 three hours, before being fixed in formalin and counted.

Potassium curiously enough exhibits a slight stimulation to division from two to three hours after treatment. Both methods

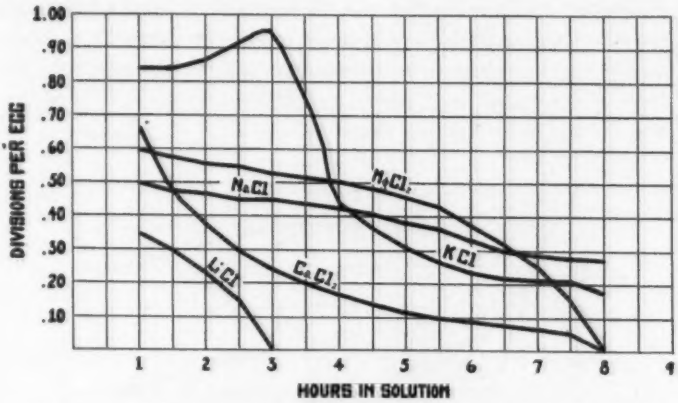

FIG. 2. The toxic effect of pure electrolytes on *Arbacia* eggs. The ordinates represent number of divisions per egg and the abscissæ represent hours in solution. Eggs were allowed to develop 1½ hours in sea water after fertilization. In Fig. 2 and Fig. 3 the number of divisions per egg has been corrected by the control.

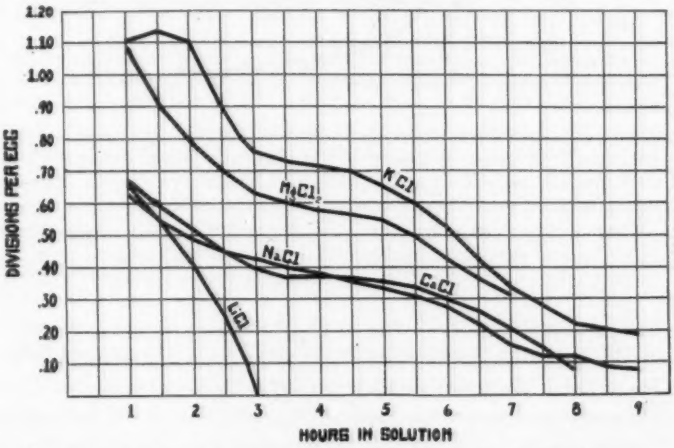

FIG. 3. Same as Fig. 2 except samples counted 3 hours after fertilization.

bring this out. $MgCl_2$ seems to be a little less toxic than $CaCl_2$ when centrifuging is not employed. We have the feeling that the toxicity of $CaCl_2$ depends somewhat on the quality of the salt used

The least toxic seems to be that of Poulenc (France). LiCl is uniformly the most toxic salt tested.

In order to show the effect of the presence of small amounts of sea water on the toxicity of the salt, eggs centrifuged three times adding fresh electrolyte solution after each centrifugation to rid them of sea water, were placed in varying mixtures of sea water and the electrolyte under investigation.

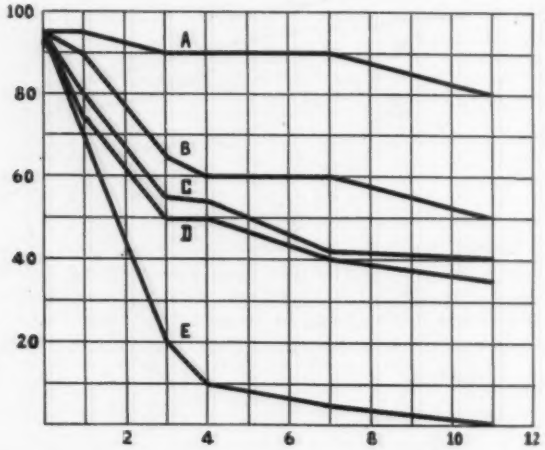

FIG. 4. Effect on toxicity of electrolytes when varying amounts of sea water are present. Ordinates represent percentage development after immersion in the electrolyte solution and abscissæ the numbers of hours of treatment. Curve *A* represents a mixture of 10 parts $CaCl_2$ plus 30 parts sea water; Curve *B* = 22 parts $CaCl_2$ plus 18 parts sea water; Curve C = 28 parts $CaCl_2$ plus 12 parts sea water; curve *D* = eggs centrifuged once from surrounding sea water and put in isotonic $CaCl_2$; curve *E* = eggs centrifuged three times with pure $CaCl_2$ to remove all trace of sea water.

The following figures (4 and 5) illustrate these results with Ca, Na and K. The other salts show similar effects. As in Fig. 1 the ordinates represent percentage of eggs which fertilized developed into the swimming stage and the abscissæ the time of treatment with the electrolyte before fertilization.

The smallest amount of sea water present reduces the salt toxicity in a most impressive manner. It makes one realize what a potent agent ionic antagonism may be.

It seems perfectly clear that the toxicity of the electrolytes as measured by the ability of the egg to be fertilized and subsequently develop, determined by two separate methods and two observers, follows the series Li > Na > Ca, Mg > K > Rb > Cs. What is not clear is the interpretation of these results.

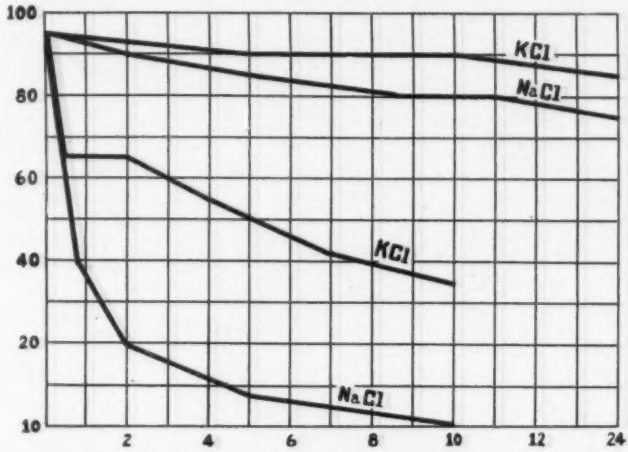

FIG. 5. Effect on toxicity of NaCl and of KCl of varying amounts of sea water Ordinates represent percentage development after immersion for the period in hours represented by the abscissæ. The two upper curves show the toxicity when the eggs are centrifuged once and placed in the isotonic salt. The two lower curves represent the toxicity when the eggs are centrifuged three times with the electrolyte.

It is suggestive that the atomic numbers, show a series very nearly identical with the order of toxicity found in this study. In order of increasing atomic number the series runs Li, Na, Mg, K, Ca, Rb, Cs.

However it is probably fortuitous that the toxicity series (with the exception of K) found for the Sea Urchin egg closely parallels the atomic number series. Any proof to the contrary must consider the action of the salts on at least the more important protein, lipoid, fat and mineral systems which constitute protoplasm and the complex interrelations of these various systems. It is possible however that this parallelism may represent the dominance of the chemical constitution of the reagent in the reaction between protoplasm and the salt.

The problem is further complicated by the fact that Chambers and Reznikoff (35, 36) have shown that the toxicity of the salts for fresh water Amœbæ immersed in the solution is

$$K > Na > Ca > Mg,$$

but when the electrolyte is injected into the interior of the protoplasm quite the reverse relation holds, viz.,

$$Ca > Mg > Na > K.$$

These results seem to implicate the chemically active plasma membrane at the surface of the cell and possibly the difference in type of protoplasm ("interior" and "exterior") as suggested by Chambers (37) and Page, Chambers and Clowes (38).

Summary.

1. The toxicity of the following cations used as chlorides for the Arbacia egg was found to be

$$Li > Na > Ca, \quad Mg > K > Rb > Cs.$$

2. It has been suggested that the term "toxicity" is applied to a miscellany of the most heterogeneous reactants in protoplasm hence as an entity its prediction and formulation in our present state of knowledge is not probable.

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